BACTERIAL DIVERSITY OF THE OCTOCORAL *PSEUDOPTEROGORGIA ELISABETHAE* VIA CULTURE-DEPENDENT AND CULTURE-INDEPENDENT METHODS AND SCREENING OF SELECTED BACTERIA FOR ANTIMICROBIAL ACTIVITY

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

In the Department of Biomedical Sciences

Faculty of Veterinary Medicine

University of Prince Edward Island

Veronica Robertson

Charlottetown, Prince Edward Island, Canada

March, 2013

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March 14, 2013

Examiners: _____
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Date: March 14, 2013

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ABSTRACT

Alcyonacean octocorals (Order Alcyonacea, amended from Order, Gorgoniidae) are both dominant on Caribbean reefs and prolific sources of bioactive natural products. Although extensive literature has been published with respect to stony coral-associated bacteria, very little is known regarding bacterial populations in healthy octocorals. In this thesis, the octocoral *Pseudopterogorgia elisabethae* was selected as a model organism to investigate owing to its commercial significance and its understudied bacterial community. The overall hypothesis of this thesis is that the bacterial community of *P. elisabethae* is diverse and can be cultured to produce a valuable and sustainable source of novel natural products. To test this hypothesis, three studies were performed. In the first investigation, pyrosequencing was used to examine the culture independent bacterial community in *P. elisabethae*. In the second study, bacteria were isolated from *P. elisabethae* using nine diverse isolation media to maximize the number of isolates. The final approach involved assessing the ability of bacterial isolates to produce antimicrobial compounds via screening fermentation extracts against five clinically relevant pathogens.

Pyrosequencing results from 14 coral-associated bacterial sequence libraries suggested *P. elisabethae* individuals are a rich source of diverse and novel bacteria. Bacterial communities were analyzed at the class and the species level, where a lack of conservation between conspecifics within and between reefs in the Bahamas was observed. From the culturing approach, 852 bacteria were isolated and were de-replicated by MALDI-TOF MS to approximately 78 unique isolates. In addition, sequences generated from de-replicated isolates were compared to the pyrosequencing community at 97% sequence similarity, yet very little overlap (0.44%) was observed. Regardless, de-replicated isolates were screened for antimicrobial activity, where a *Streptomyces* sp. RKBH-B54 and genetically-related *Pseudoalteromonas* spp. were reproducibly bioactive against yeast and Gram-positive bacteria respectively. Due to the potent bioactivity of RKBH-B54, a bioassay guided fractionation was performed in an attempt to isolate the antifungal compound produced. Dihydromaltophilin and maltophilin were proposed as the most likely compounds responsible for the observed antifungal activity from RKBH-B54 based on comparison of UPLC/HRMS data to analytical databases; unfortunately, the instability of these compounds prevented complete purification and structural elucidation. In conclusion, this thesis has demonstrated that *P. elisabethae* is a rich source of diverse bacteria, yet collecting multiple individuals throughout the Bahamas is required to truly evaluate the microbial biodiversity in this octocoral. It is also concluded that the potent and reproducible bioactivity of selected bacterial extracts from *P. elisabethae* can be used as a resource for developing potentially new therapeutic agents.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AGE	Agarose gel electrophoresis
bTEFAP	bacterial tag-encoded FLX-amplicon pyrosequencing
BLAST	Basic Local Alignment Search Tool
BS	Bahamas
CHCA	alpha cyano-4-hydroxycinnamic acid
CTAB	Cetyl trimethyl ammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELSD	Electron light scattering device
EtOAc	Ethyl acetate
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IC_{50}	Half maximal inhibitory concentration
LCMS	Liquid chromatography mass spectrometry
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MeOH	Methanol
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance

OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
ppm	parts per million
Ps	Pseudopterosin(s)
RDP-II	Ribosomal database project
RP-HPLC	Reverse-phased high performance liquid chromatography
RTL	Research & Testing Laboratories
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SEP(s)	*Spongiobacter*/*Endozoicomonas* phylotype(s)
UV-Vis	Ultraviolet visible (spectroscopy)
UPLC	Ultra-high performance liquid chromatography
SCUBA	Self-contained underwater breathing apparatus
VRE	Vancomycin resistant *Enterococcus*

CHAPTER I

INTRODUCTION AND BACKGROUND

CORAL REEFS

Coral reefs are an integral part of the well-being of the oceans, and are of major economic benefit to many countries (Reshef *et al*., 2006). Ranging from tropical, subtropical, and deep-sea ecosystems, coral reefs cover only 0.2% of the world's oceans (Wood, 1998), yet their unsurpassed biological diversity have rendered them to be affectionately called "the rainforests of the sea" (Connell, 1978; Reaka-Kudla, 1997). As with rainforests, the value of reefs results from the millions of species that live primarily or exclusively within these habitats (Knowlton, 2001a). Ecologically, coral reefs serve as a physical barrier to protect coastlines and communities from wave action and erosion (Wood, 1998). Reefs also assist in nutrient recycling, and play a vital role in adding to the marine food chain via their symbiotic algae (Baker, 2003). Furthermore, they provide valuable habitats for an estimated nine million marine species (Knowlton, 2001b) including fish (Sale, 1993), invertebrates, (Kaplan *et al*., 1999; Knowlton and Rohwer, 2003) and microorganisms (Wood, 1998). Economically, an estimated US$375 billion each year is generated directly or indirectly by coral reefs through employment opportunities, food, the sale of aquarium fish, and tourism (Costanza *et al*., 1997).

Corals and other invertebrates have received much attention from academic and industrial groups, stemming from the biomedical potential of their chemical extracts. Without physical protection or effective escape mechanisms, sessile or slow moving organisms, including corals, are likely to be protected through chemical defence (Noyer *et al*., 2011). These defences, in the form of natural products, provide a resource for drug discovery (Harvey, 2000; Noyer *et al*., 2011) and can become clinically used drugs or can act as scaffolds for semi-synthetic drugs

(Vizcaino, 2011). The biomedical applications of marine-derived extracts are numerous and have recently been reviewed (Butler, 2008; Cragg and Newman, 2005; Newman and Cragg, 2004; Proksch *et al*., 2002). Selected examples of marine-derived natural products which have been approved by the US Food and Drug Administration (FDA) or are in clinical trials include treatments for cancer, inflammation, pathogenic microorganisms, pain, and asthma.

ALCYONACEAN OCTOCORALS

The subclass Octocorallia (Order: Alcyonacea; Bayer, 1961) includes over 3000 species of soft corals, sea fans, and sea pens (McFadden *et al.*, 2010). By definition, octocorals are monophyletic cnidarians with polyps containing eight feather-like tentacles and eight internal mesenteries (McFadden *et al*., 2010; Sánchez *et al*., 2003), and with only one known exception (*Fungia* sp., Coll, 1992), all octocorals are colonial (McFadden *et al.*, 2010). Previously classified as gorgonian octocorals (Order: Gorgonidae) (Bayer, 1961), this terminology has recently been discredited by octocoral taxonomists, as the order was defined solely on the basis of differences in colony growth morphology (McFadden *et al*., 2010). Molecular evidence now classifies all "gorgonians" under the order Alcyonacea (McFadden *et al*., 2006; McFadden *et al*., 2010).

Abundant on Atlantic and Pacific reefs, octocorals represent almost 40% of the known fauna in the Caribbean, with more than 195 species reported (Fenical, 1987; Penn *et al*., 2006). The ability of octocorals to deter predators via chemical defence may in part explain their dominance on Caribbean reefs (Whalen *et al*., 2010).Sessile life styles, slow growth rates and soft-bodied morphologies has likely resulted in a selection for a vast arsenal of bioactive chemicals with which to defend themselves. In view of their ecological dominance on coral reefs, alcyonacean octocoral chemistry has been thoroughly investigated, revealing a wealth of

chemically diverse, bioactive metabolites (reviewed in Berrué and Kerr, 2009). Selected coral-derived bioactive metabolites are highly significant prospects in clinical trials (Berrué *et al*., 2011a); however, the progression of some of these biologically active compounds through clinical trials has been hindered by limited supplies (Berrué *et al*., 2011b).

THE OCTOCORAL *PSEUDOPTEROGORGIA ELISABETHAE*

Members of the genus *Pseudopterogorgia* are the most abundant octocoral in the Caribbean, with over 15 species described (Heckrodt and Mulzer, 2005). As one example, the sea plume *Pseudopterogorgia elisabethae* (Octocorallia; Bayer, 1961, Figure 1.1) has a moderate distribution in the West Indies and resides in tropical forereefs at 5 – 35 m (Gutierrez- Rodriguez *et al*., 2009; Heckrodt and Mulzer, 2005). Morphologically, *P. elisabethae* consists of a cluster of feather-like branches, each with a central skeleton with numerous, closely spaced branchlets which extend from the central skeleton (Guiterrez-Rodriguez and Lasker, 2004). Colonies of this alcyonacean demonstrate significant genetic and morphologic variation within and among populations in the Caribbean Sea (Gutiérrez-Rodríguez and Lasker, 2004; Gutiérrez-Rodríguez *et al*., 2009), and demonstrates a higher inter-individual genetic variation than most other corals (7.3% compared to up to 3.5%; Gutiérrez-Rodríguez *et al*., 2009). As with many other shallow water octocorals, *P. elisabethae* contains symbiotic algae called zooxanthellae, with current taxonomic resolutions suggesting the coral is exclusively hosting *Symbiodinium* sp. clade B (Santos *et al*., 2003). *P. elisabethae* colonies are subject to grazing only by specialized predators, most notably by the flamingo tongue snail *Cyphoma gibbosum*, as well as by the polychaetes *Hermodice* sp. and the four-eyed butterfly fish *Chaetodon capistratus* (Castanaro and Lasker, 2003; Thornton and Kerr, 2002).

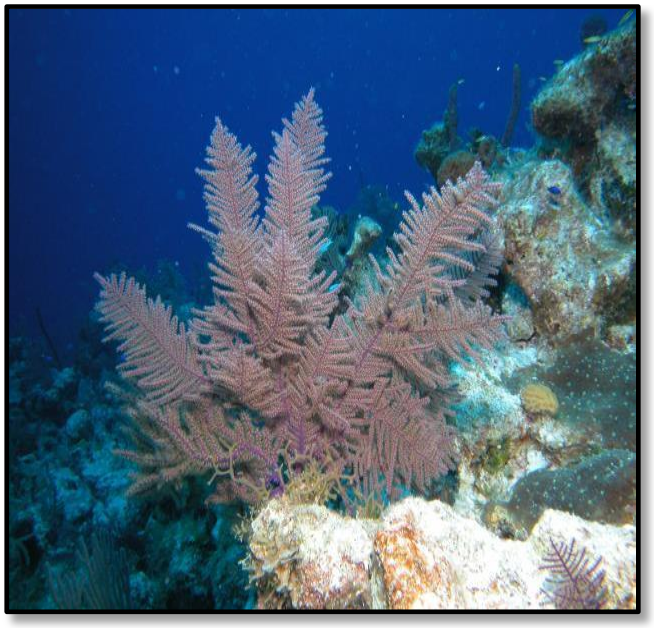


Figure 1.1: The octocoral *Pseudopterogorgia elisabethae* (Photo credit: R. Kerr).

PROKARYOTIC DIVERSITY

Prokaryotes constitute the most biologically diverse kingdom on Earth (Rohwer *et al*., 2002; Sunagawa *et al*., 2010), with abundance estimates ranging up to 10^{30} species (Pedros-Alio, 2006). Although prokaryotes occupy almost every niche on the planet (von Wintzingerode *et al*., 1997), only 6000 bacterial species have received a formal description to date, compared with a formal description for more than a million plants and animals (Harvey, 2000; Pedros-Alio, 2006). Traditionally, prokaryotic biodiversity has been measured using conventional approaches, including sampling, counting, and cultivation to axenic (pure) cultures (Jones, 2007). In microbial cultivation, the general approach is to provide nutrients similar to the natural environment with which to grow the target species (Sipkema *et al*., 2011), yet successful cultivation appears to be dependent on specialized techniques (Ben-Dov *et al*., 2009; Button *et al*., 1993; D'Onofrio *et al*., 2010; Kaeberlein *et al*., 2002; Morris *et al*., 2008; Sipkema *et al*., 2011), including prolonging incubation times (Davis *et al*.,2005), using alternative gelling agents in solid media (Tamaki *et al*., 2009), co-cultivation of interdependent organisms (Muyzer and Smalla, 1998), and sample pre-treatment (Jensen *et al*., 2005). However, out of over 40 known prokaryotic phyla, only half have cultured representatives based on comparison with 16S rRNA gene sequences (Connon and Giovannoni, 2002), where estimates suggest only 0.01 – 0.1% of cells may be cultured using today's technology (Amann *et al*., 1995; Kogure *et al*., 1979). Consequently, the great majority of bacteria have not been evaluated for their pharmacological potential (Manyak and Carlson, 1999), nor have they been described (Staley, 2006). This phenomenon is commonly referred to as the "great plate count anomaly," as coined by Staley and Konopka (1985) and describes the difference between the number of organisms cultivated on standard agar media plates from those countable by microscopic examination (Connon and

Giovanni, 2002). Regardless, cultivation studies are still relevant and vitally important in drug discovery today as biomedically active drugs or drug candidates from microbial origin such as penicillin are generated by continuous fermentation (Johnson, 1952). As medicines of microbial origin have generated worldwide revenues of roughly $83 billion for the pharmaceutical industry (Manyak and Carlson, 1999), screening efforts for unique, novel bacteria provide a significant and untapped resource for chemical diversity and drug discovery programs (Manyak and Carlson, 1999).

Several theories exist to explain the "great plate count" paradigm. For instance, growth of microbial species may require oligotrophic conditions to mimic their natural environment; oftentimes this is not provided in nutrient rich laboratory media containing complex carbon sources (Connon and Giovanonni, 2002). These unnatural growth conditions expose the organisms to a variety of potential environmental stressors, often rendering them unable to grow on solid media (Olson *et al*., 2000). Furthermore, many oligotrophic bacteria grow slowly and to low cell densities, and thus their presence can be overlooked in culture libraries (Simmons *et al*., 2008). As the doubling times of some microorganisms can be up to 100 days (Kaeberlain *et al*., 2002), patience in laboratory experiments is necessary, since this time frame is much longer than most reported cultivation studies (Sipkema *et al*., 2011). In addition, microorganisms with fast growth rates are often detected on standard agar (Kirk *et al*., 2004), which are rarely the most dominant species in their habitat (Hugenholtz, 2002). Although cultivation is a requirement to officially name and characterize a bacteria (Button *et al*., 1993; Pedros-Alio, 2006), it is important to note that provisional nomenclature of organisms may be granted if their growth is not yet possible in the laboratory (Staley, 2006). This is permissible only if sufficient additional information is known, including their molecular guanine-cytosine (GC) percent content, their

16S rRNA gene sequence, cell size and cell shape, and are thus termed "Candidatus" organisms (Staley, 2006).

Since the inception of utilizing the16S rRNA gene (Woese and Fox, 1977) by Pace and colleagues in PCR-generated prokaryotic biodiversity analyses (Pace *et al*., 1985), analysis of environmental prokaryotes have more than tripled the number of identifiable bacterial phyla (Hugenholtz *et al*., 1998) and has revolutionized our current understanding of bacterial diversity (Kirk *et al*., 2004). Currently, the 16S rRNA gene sequence constitutes the largest gene-specific database for measuring prokaryotic diversity, and the number of entries in easily accessible databases is continuously increasing (Dieckmann *et al*., 2005). This method of determining prokaryotic diversity is referred to as a culture-independent approach, which identifies prokaryotes on the basis of their nucleic acid sequence. Although bacterial systematics has not yet reached a general consensus for describing bacteria at the species level (Cohan, 2002), taxonomists have generally agreed to define a species on the basis of a DNA-DNA re-association value of greater than 70% (Amann *et al*., 1995), corresponding to a circa 97% 16S rRNA gene sequence similarity (Stackebrandt and Gobel, 1994). Today, culture-independent approaches form the backbone of many publications regarding bacterial populations in humans, invertebrates, soil, sediment, plants, and microorganisms.

The marine environment represents a relatively unexplored environment for microbial cultivation and subsequent drug discovery. Prokaryotes have been evolving over the past 3.5 billion years, which is presumed to have resulted in exceptionally high levels of genetic and phenotypic diversity (Sogin *et al*., 2006). With the great diversity of bacteria and with genes adapted to a wide range of habitats (from deep sea trenches, to hydrothermal vents, to various marine invertebrates), a wide variety of unique secondary metabolites may be produced which

can significantly increase the diversity of natural product screening libraries (Harvey, 2000). Furthermore, by finding a microbial producer of biomedically important compounds, fermentation technologies can provide a continuous and sustainable source of supply to meet the demands required for human clinical trials. As prokaryotes from unique marine habitats have largely been unexplored with respect to their biomedical potential, this demonstrates that marine prokaryotes offer a wealth of diversity remaining to be exploited in clinical applications.

THE CORAL HOLOBIONT

Stony and alcyonacean corals associate with a wide variety of microorganisms, including algae (termed dinoflagellates or zooxanthellae; Cantin *et al*.,2009; Goulet and Coffroth, 2004; Mieog *et al*., 2009; Voolstra *et al*., 2009), bacteria (Bourne and Munn, 2005; Brück *et al*., 2007; Rohwer *et al*., 2001, 2002; Rosenberg *et al*., 2007), fungi (Bentis *et al*., 2000; Wang *et al*., 2011), and only in the case of stony corals known so far, archae (Wegley *et al*., 2009) and virus-like particles (Marhaver *et al*., 2008; van Oppen *et al*., 2009). These associations are generally believed to be of symbiotic nature (Shnit-Orland and Kushmaro, 2009), and are collectively referred to as the coral holobiont (Rohwer *et al*., 2002). Owing to their high microbial diversity, coral associated microbes are of particular interest to both microbial ecologists and natural products chemists by offering a potentially superior source of unique, novel chemistries for biotechnological applications (Jackson *et al*., 2012). Furthermore, so delicate is the balance between corals and their associated microbes is that disease and bleaching of both scleractinians and octocorals (a term which describes the visible whitening of corals when symbiotic dinoflagellates are expulsed; Ben-Haim *et al*., 1999) has been known to occur when the corals are infected by opportunistic fungal (Alker *et al*., 2001; Rypien *et al*., 2008) or bacterial (Ben-Haim *et al*., 2003; Kushmaro *et al*., 2001; Vizcaino *et al*., 2010) pathogens.

CORAL-ASSOCIATED BACTERIAL DIVERSITY

Scleractinian-associated bacterial diversity. As mentioned previously, scleractinian corals are host to a diverse array of bacteria which may be symbiotic or pathogenic in nature. Although prokaryotic diversity has traditionally been measured using cultivation approaches (Torsvik and Øvreås, 2002), up to 99.8% of bacteria cannot be cultured by current isolation techniques (Koren and Rosenberg, 2006). This paradigm has been offset by targeting the 16S rRNA gene, which bypasses cultivation by directly accessing prokaryotic nucleic acids. Investigation of scleractinian coral-associated bacterial diversity using the small subunit rRNA gene sequence began in 2001, where diversity assessments targeting the 16S rRNA gene revealed bacteria form diverse, species-specific associations within the stony coral holobiont (Frias-Lopez *et al*., 2003; Koren and Rosenberg, 2006; Rohwer *et al*., 2001; 2002; Rosenberg *et al*., 2007). In addition, 16S rRNA gene sequence analysis of the bacterial communities associated with the stony corals *Montastrea* sp., *Porites* sp., *Diploria* sp. and *Pocillopora* sp. revealed bacterial communities were unique for each coral species analyzed (Bourne and Munn, 2005; Rohwer *et al*., 2002).

Within the scleractinian coral holobiont (Rohwer *et al*., 2002) bacterial communities represent the highest phylogenetic diversity and are one of the most numerically dominant microorganisms present (Wegley *et al*., 2007) with an estimated $10^5 – 10^6$ bacteria per mL (Reshef *et al*., 2006). Although numerous and diverse, the role of most coral-associated bacteria within the coral holobiont remains largely unknown, as definitive roles can only be hypothesized based on difficulties in bacterial cultivation (Reshef *et al*., 2006). However, probiotic roles have been proposed in hard corals (Reshef *et al*., 2006), along with providing the host with nutrients such as nitrogen (Lema *et al*., 2012; Lesser *et al*., 2004) and carbon (Wegley *et al*., 2007), while

sulphur-oxidizing bacteria may remove metabolic waste products generated from the host invertebrate (Webster *et al*., 2001). Furthermore, particular classes of coral-associated bacteria provide antibiotic protection from invasive pathogens by producing bioactive metabolites, which are believed to aid in host immune defense (Jackson *et al*., 2012; Ritchie, 2006). Scleractinian coral-associated bacteria are found in all layers from the surface mucopolysaccharide layer (Ducklow and Mitchell, 1979; Rohwer *et al*., 2001), to the tissue (Nithyanand and Pandian, 2009), and to the skeleton (Rosenberg *et al*., 2007; Sweet *et al*., 2011), and each of these habitats harbour distinct bacterial species (Bourne and Munn, 2005; Koren and Rosenberg, 2006; Sweet *et al*., 2011). In addition, stony coral-associated bacterial communities are shaped by a broad variety of biotic and abiotic factors. Abiotic factors which influence biodiversity include geographic distance between coral populations (Littman *et al*., 2009b), seawater pH (Meron *et al*., 2011), rainfall (Chen *et al*., 2010) and season (Ceh *et al*., 2011; Chen *et al*., 2010; Koren and Rosenberg, 2006); biotic factors affecting diversity include disease (Bourne *et al*., 2009) and competitive interactions from other microbes (Torsvik and Øvreås, 2002). Although substantial evidence suggests bacterial assemblages may be specific in hard corals (Rohwer *et al*., 2001, 2002; Rosenberg *et al*., 2007); contrasting evidence suggests cnidarian microbiology is more complex and transient than previously assumed. Microbial communities have been shown to differ between sites (Kellogg *et al*., 2009; Kvennefors *et al*., 2010), colour morphotypes (Neulinger *et al*., 2009), conspecifics (Chen *et al*., 2010), life stages of the coral (Littman *et al*., 2009a), and spatial locations within the cnidarian holobiont (Bourne and Munn, 2005; Qian *et al*., 2006; Schöttner *et al*., 2009; Sweet *et al*., 2011; Wichels *et al*., 2006). For example, acroporids collected in triplicate from two locations in the Great Barrier Reef revealed that bacterial communities grouped according to location rather than species (Littman *et al*., 2009b).

Furthermore, bacterial communities of triplicate stony coral libraries not only showed bacterial variation within the same environment, but also across locations and by coral colour morph (Neulinger *et al*., 2008). It is generally well-accepted that bacteria present in scleractinian corals are distinct from those in the surrounding water column (Bourne and Munn, 2005; Jackson *et al*., 2012; Kvennefors *et al*., 2010; Rohwer *et al*., 2002; Sunagawa *et al*., 2010).

Octocoral-associated bacterial diversity. Microorganisms are an integral part of contributing to the health of scleractinian corals. However, despite the growing recognition that scleractinians live in a symbiotic relationship with a diverse array of bacteria and despite the advancement of sequencing technology, studies characterizing the microbial diversity of octocorals are rare. To date, only five studies on octocorals exist (Brück *et al*., 2007; Gray *et al*., 2011; Penn *et al*., 2006; Sunagawa *et al*., 2010; Webster and Bourne, 2007) and only one study has characterized a shallow-water octocoral-associated bacterial diversity using next generation pyrosequencing methods (Sunagawa *et al*., 2010). For example, Webster and Bourne (2007) profiled the bacterial community structure associated with the Antarctic alcyonacean octocoral *Alcyonium antarcticum*. Here, bacterial communities were analyzed from corals collected at three sites in McMurdo Sound, Antarctica. Both molecular and cultivation approaches revealed coral-associated Gammaproteobacteria were conserved within and between sites, while 16S rDNA sequences demonstrated low sequence similarity to previously published sequences in the GenBank database (Webster and Bourne, 2007). Conclusions drawn from this study supported the theory that at least some alcyonacean corals harbor unique bacterial inhabitants (Webster and Bourne, 2007). Some studies also suggest octocoral-associated bacteria vary between sites (Gray *et al*., 2011; Penn *et al*., 2006) and octocoral genera (Brück *et al*., 2007). Relative proportions of the dominant bacterial phyla are variable in some octocorals (Gray *et al*., 2011; Penn *et al*.,

2006). For example, Gray *et al.* (2011) used clone libraries of 16S rRNA gene sequences to reveal deep-sea octocoral-associated bacterial consortia differed between conspecifics collected at different sites in the Aleutian Islands, where a variable bacterial community was observed. Here, two clone libraries were dominated by the phylum Tenericutes while Alphaproteobacteria was the dominant class for the remaining libraries. Furthermore, other clone libraries were more similar at the genus level rather than within conspecifics.

Rationale for studying coral-associated bacteria. Understanding coral-associated microbial biodiversity is critical for defining ecological networks (Brück *et al*., 2007), characterizing resilience to disease (Bourne and Munn, 2005; Fonseca *et al*., 2010), providing effective reef management (Sharp *et al*., 2010), and understanding co-evolution of cnidarians with their microbial inhabitants (Pedros-Alio, 2006). Interestingly, a growing body of literature suggests bacteria have co-evolved with their cnidarians hosts, forming species-specific clades which are highly divergent from one another (Jackson *et al*., 2012; Lee *et al*., 2009; Webster *et al*., 2010). This fascinating discovery suggests cnidarian-associated bacteria may be uniquely adapted to highly specialized niches. In particular, corals can provide a high nutrient niche suitable for bacterial colonization within the oligotrophic ocean (Ducklow and Mitchell, 1979; Sharon and Rosenberg, 2008). From a microbial point of view, the coral host can be viewed as a unique microhabitat to occupy, and therefore, can be a site for highly adapted species (Amann *et al*., 1995). These well-adapted species have evolved under the pressures of natural selection to a particular microhabitat (Noyer *et al*., 2011). Based on preliminary evidence that some natural products, previously believed to be of octocoral origin, may be produced by symbiotic bacteria (Bunyajetpong *et al*., 2011; Bunyajetpong, 2011) and owing to the difficulties in harvesting marine invertebrates, microorganisms are very attractive to researchers and pharmaceutical

companies alike. As alcyonacean octocorals are largely unexplored and unique habitats, this microenvironment is ideally suited for exploration of their biomedical potential via their associated bacterial community (Singh and Pelaez, 2008). As such, cultivating marine invertebrate microbial symbionts represents a pivotal step forward towards generating consistent and commercial yields available for supplying invertebrate-derived compounds by continuous fermentation (Fortman and Sherman, 2005). By focusing research efforts on novel and unique habitats – including the octocoral microhabitat – one can investigate these specialized habitats for their potential to provide novel natural products.

METHODS OF ASSESSING CORAL-ASSOCIATED BACTERIAL DIVERSITY

Clone libraries. Genetic analysis of microbial communities is often complex and daunting due to the great complexity of such systems. As such, a number of PCR-based approaches have been developed to characterize microbial diversity, including clone libraries (Scharf *et al*., 1986), automated ribosomal intergenic spacer analysis (ARISA), fluorescence *in situ* hybridization (Ainsworth *et al*., 2006), temperature gradient gel electrophoresis (TGGE), Box-PCR (Lanoot *et al*., 2004), and denaturing gradient gel electrophoresis (DGGE) (Kirk *et al*., 2004; Muyzer *et al*., 1993). These techniques, called community fingerprinting techniques, all provide a "snapshot" of the overall structure and diversity in microbial populations (Rettedal *et al*., 2010). For example, clone libraries using 16S rRNA gene sequences have provided the foundation for most coral-associated bacterial community studies following their introduction by Scharf and colleagues in 1986 (Scharf *et al*., 1986). The premise of clone libraries involves separation of PCR-amplicons (targeting a particular gene sequence, ex. the 16S rRNA gene) from one another by cloning in competent *E. coli* cells (von Wintzingerode *et al*., 1997). The amplicons are ligated (i.e. inserted) into a plasmid vector, the latter of which often contains a

gene sequence which is lethal to *E. coli* when expressed (yet is disrupted by ligation of a DNA insert into the cloning site) and antibiotic resistance genes (Timmis *et al*., 2010). Cells are plated onto a selective medium with antibiotics added; therefore, only cells which have successfully been transformed can grow. Furthermore, the need for tedious blue/white screening is eliminated with plasmids encoding for a gene lethal to *E. coli*, as cells which have not been transformed still express the lethal gene and subsequently causes death of *E. coli* when plated. However, due to the biases, cost, and labor-intensities of constructing clone libraries (von Wintzingerode *et al*., 1997), other techniques to describe microbial communities have been explored.

Denaturing gradient gel electrophoresis. Arguably the most commonly used genetic fingerprinting technique (Rettedal *et al*., 2010), denaturing gradient gel electrophoresis (DGGE) was applied to the field of estimating microbial diversity by Muyzer *et al.* (1993) and has since been applied to profiling bacterial communities in hot springs (Ferris *et al*., 1996), reef-building corals (Bourne and Munn, 2005), soil (reviewed in Kirk *et al*., 2004), and sponges (Webster *et al*., 2004). The basic principle in DGGE is that a mixture of similar sized DNA amplicons, each containing different nucleotide sequences, will migrate to unique positions in a polyacrylamide gel according to their melting properties (Muyzer *et al*., 1993; Muyzer and Smalla, 1998; Sheffield *et al*., 1989). Here, double stranded DNA amplicons are electrophoresed through a linear gradient of DNA denaturants (formamide and urea) (Muyzer *et al*., 1993; Sheffield *et al*., 1989). Upon reaching a particular denaturing concentration, equal to the melting temperature of the nucleotide sequence with the lowest melting point, the double stranded DNA partially dissociates, the fragment halts, and discrete bands are formed in the gel at unique positions (Ferris *et al*., 1996; Muyzer and Smalla, 1998; Sheffield *et al*., 1989). Each band represents one gene sequence from a microorganism (Kirk *et al*., 2004). DNA bands may then be stained

(Muyzer and Smalla, 1998), excised, and the nucleotide sequences can be determined (Ferris *et al*., 1993).

The main feature of DGGE is that sequences differing by a single base pair can be separated, as single base substitutions alter the melting properties of the DNA sequence strongly enough to separate molecules (Myers *et al*., 1985). Sequence specific strand separation from DNA amplicons of identical size is guaranteed by the addition of a 35 – 40 base pair GC-rich motif – called a GC clamp – to the 5' end of one of the primers during PCR amplification (Ferris *et al*., 1996; Kirk *et al*., 2004; Muyzer *et al.*, 1993). The GC clamp ensures at least part of the DNA remains double stranded during denaturation on the denaturing gel (Ferris *et al*., 1996; Kirk *et al*., 2004; Muyzer *et al*., 1993); failure to do so will result in DNA denaturing completely into single strands (Kirk *et al*., 2004). Although DGGE is fast, reliable, reproducible, and cost-effective, it is limited in a variety of ways (reviewed in Kirk *et al*., 2004). For example, only sequences constituting higher than 1 – 2% of the population present can be detected (Kirk *et al*., 2004; Muyzer *et al*., 1993). In addition, the GC clamp sequence can cause incomplete strand synthesis during PCR amplification leading to multiple bands for one template (von Wintzingerode *et al*., 1997). Furthermore, multiple sequences with minor differences can migrate to identical positions in DGGE gels and may not be separated in a mixed sample; therefore, one band may not represent one species (Jackson *et al*., 2000). Due to such limitations of DGGE and as reviewed elsewhere (Kirk *et al*., 2004), other methods of assessing culture-independent microbial diversity have been developed, including next generation sequencing methods described below.

Pyrosequencing. In 1998, a new sequencing technology was introduced (Ronaghi, 1998) which would change the nature of bacterial research (Margulies *et al*., 2005). This second-generation sequencing technology, termed pyrosequencing, has allowed for the identification of rare and novel microbial phylotypes by unparalleled sequencing depth compared to traditional methods (Amend *et al*., 2010; Sunagawa *et al*., 2010), which are well understood to underestimate diversity by 1 – 2 orders of magnitude (Engelbrektston *et al*., 2010; Uthicke and McGuire, 2007). Pyrosequencing, a term coined from the use of pyrophosphate-based sequencing (Margulies *et al*., 2005) is a fully automated sequencing technology capable of generating over 25 million bases in a single 10-hour run, and is performed with ≥99.75% accuracy (Huse *et al*., 2007; Lee *et al*., 2011; Margulies *et al*., 2005). This process has been scaled up by several laboratories, including 454 Life Sciences and Research and Testing to be massively parallel, generating more than 400,000 sequence reads per plate (Quince *et al*., 2009) for roughly 10 – 30 times cheaper than Sanger sequencing (Edwards *et al*., 2006). In contrast to traditional Sanger sequencing, pyrosequencing technology does not require a cloning step, thus avoiding potential biases discussed elsewhere (Edwards *et al*., 2006). In assessing microbial communities, DNA is extracted, and in the case of general bacterial sequencing, the 16S rRNA gene sequence is amplified by PCR prior to pyrosequencing, although it is possible to directly pyrosequence genomic DNA to mitigate potential biases and sequencing errors from multiple rounds of PCR. Based on a "sequencing-by-synthesis" technology, pyrosequencing relies on the light detection of inorganic pyrophosphate (PPi) released upon every nucleotide incorporation during DNA synthesis (Ronaghi *et al*., 1998). To begin, each of the four nucleotide triphosphates are cycled over the plate. If the first unpaired base in a well is complementary to the incoming nucleotide, then DNA polymerase (Quince *et al*., 2009) catalyzes the reaction to ultimately

release light (Edwards *et al*., 2006; Margulies *et al*., 2005; Ronaghi *et al*., 1998). The amount of light produced in the luciferase-catalyzed reaction is recorded with a CCD (charge - coupled device) camera, which captures emitted photons (Margulies *et al*., 2005). The pattern of light intensities emitted can be used to report the order of nucleotide incorporations, and thereby reflect the DNA sequence reads in an orderly manner (Huse *et al*., 2007; Quince *et al*., 2009; Ronaghi *et al*., 1998). Finally, the genetic variation within the sequence reads can be used to assess the phylogenetic diversity of the bacterial community within a sample (Quince *et al*., 2009). In prokaryotic analysis, read lengths of ~430 bp allows for sequence resolution to the genus level based on pyrosequencing of the hypervariable V1 – V3 region of the 16S rRNA gene (Jackson *et al*., 2012).

Recently, bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP; Dowd *et al*., 2008) has been introduced as a powerful molecular tool to rapidly characterize prokaryotic bacterial communities in a higher sequencing depth than traditional 16S rDNA clone libraries. In 2006, the Rohwer laboratory (Edwards *et al*., 2006) was the first to apply pyrosequencing methods to environmental samples, indicating that this technology is still relatively a new approach to assessing bacterial diversity in environmental habitats. Furthermore, Lee *et al.* (2011) compared pyrosequencing to traditional clone library methods, where the authors discovered six additional bacterial phyla in sponge pyrosequencing 16S libraries which were not detected in traditional 16S rDNA clone libraries (Lee *et al*., 2011). These phylotypes were detected in very low numbers and would most likely have gone unrecognized by traditional culture-independent analyses. Likewise, pyrosequencing revealed novel sponge phylotypes which were not detected in the surrounding seawater (Webster *et al*., 2010). Presently, bTEFAP has been successfully implemented in characterizing bacterial communities in fermented seafood

(Roh *et al*., 2010), cattle feces (Dowd *et al*., 2008), sponges (Webster *et al*., 2010), and soil (Acosta-Martínez *et al*., 2008). The large sequence yield generated from pyrosequencing has exponentially increased our ability to explore rare and novel phylotypes within environmental samples (Kirchman *et al*., 2010; Sogin *et al*., 2006). In traditional studies, dominant cultures or phylotypes have masked the detection of low abundance sequences (Sogin *et al*., 2006). However, the majority of genetic diversity revealed by second generation sequencing in the North Atlantic Sea was represented by thousands of low abundance sequence populations (Sogin *et al*., 2006). This "rare biosphere" (Sogin *et al*., 2006) has a remarkable source of genomic variation, and is a largely underexplored potential reservoir of biological diversity (Sunagawa *et al*., 2010).

DEREPLICATION OF CULTIVABLE BACTERIAL LIBRARIES

As mentioned previously, the ideal approach in bacterial cultivation is to provide nutrients and a habitat similar to the natural environment with which to grow the target species (Sipkema *et al*., 2011). During the isolation process, several hundreds to several thousands of environmental strains can be easily accumulated (Singh and Pelaez, 2008). In order to discriminate between potentially interesting, unexplored groups of bacteria, it is essential to minimize the number of redundant or previously investigated isolates in a sample library (Dieckmann *et al*., 2005; Singh and Barrett, 2006). In the context of drug discovery, this process – the removal of knowns from unknowns at user defined taxonomic levels generally from genera to species – is called dereplication, and it is critical to success in reducing labor intensity, time, and screening costs (Dieckmann *et al*., 2005; Ghyselinck *et al*., 2011). This aids in the focusing of efforts on characterizing selected, novel, or rare microorganisms for downstream applications, including drug discovery.

Molecular methods. Several methods for bacterial dereplication exist, including the use of repetitive extragenic palindromic (REP) elements and subsequent PCR amplification (Rep-PCR) (Versalovic *et al*., 1991), enterobacterial repetitive intergenic consensus (ERIC) sequences, restriction fragment length polymorphism (RFLP), Box-PCR (Lanoot *et al*., 2004), randomly amplified polymorphic DNA (RAPD) (Lynch and Milligan, 1994), and fatty acid methyl ester (FAME) (Haack *et al*., 1994) analyses. Each of these techniques, including their description, advantages, and disadvantages, have been reviewed (Kirk *et al*., 2004). REP and ERIC sequences are invaluable tools for determining genetic variation among Gram-negative bacteria (Versalovic *et al*., 1991), where the former elements act as regulatory elements within the untranslated region of the transcriptome (Versalovic *et al*., 1991). However, REP elements and ERIC sequences do not concern Gram-positive bacteria, as probes for REP and ERIC sequences hybridized preferentially to genomic DNA from Gram-negative bacteria, but failed to yield hybridization signals for Gram-positive bacteria (Versalovic *et al*., 1991). However, the BOX element has been developed as a tool for discriminating between closely related species in Gram- positive bacteria (Martin *et al*., 1992). The BOX elements are composed of three subunits (boxA, boxB, and boxC) which are 45 – 59 bases long, and are highly conserved regulatory elements found in Gram-positive bacteria (Martin *et al*., 1992). However, although BOX-PCR has been used to discriminate bacteria to the strain level, it is unable to reliably differentiate some bacteria to the species level (Kim *et al*., 2002). Furthermore, none of these technologies can simultaneously dereplicate Gram-positive and Gram-negative bacteria. Hence, in those instances where it is vital to differentiate to the species level and to differentiate between both Gram- negative and Gram-negative bacteria, new techniques are required.

MALDI-TOF MS. Recently, matrix-assisted laser-desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has been introduced as a powerful, reliable tool to dereplicate a wide variety of microorganisms, with most efforts focusing on clinically relevant microbial pathogens (Fenselau and Demirev, 2001), fungi (Neuhof *et al*., 2007), as well as both Gram-positive and Gram-negative bacteria (Vargha *et al*., 2006). MALDI-TOF MS has been routinely demonstrated to differentiate from species to sub-strains in clinical isolates (Bizzini and Greub, 2010; Carbonnelle *et al*., 2011; Mellman *et al*., 2008; Vanlaere *et al*., 2008; Vargha *et al*., 2006) and more recently, environmental bacteria from marine invertebrates (Dieckmann *et al*., 2005). Developed in the 1980s, MALDI-TOF MS uses a "soft" ionization technique, which induces only a small amount of molecule fragmentation (Schiller *et al*., 2007). Briefly, following the growth of pure cultures, individual colonies are spotted onto a stainless steel target plate and mixed with a photon-absorbing organic matrix, where the sample and the matrix form a co-crystallized mixture (Fenselau and Demirev, 2001). Ionization of whole microbial cells (Dieckmann *et al*., 2005; Neuhof *et al*., 2006), which is particularly valuable in allowing for minimal sample preparation, is generated by the addition of a strong acid (generally trifluoroacetic acid, TFA) to the MALDI matrix (Liu *et al*., 2007). A laser converts the molecules in the crystallised sample-matrix mixture into gas-phase ions (Fenselau and Demirev, 2001), which are generally pulsed into a time-of-flight tube (Carbonnelle *et al*., 2011; Fenselau and Demirev, 2001; Vanlaere *et al*., 2008), where ions are separated by molecular weight after migration in an electric field (Carbonnelle *et al*., 2011). The mass-to-charge ratio (m/z) of an analyte is plotted against intensity by the mass spectrometer (Carbonnelle *et al*., 2011; Vanlaere *et al*., 2008), resulting in a computer-generated spectral output from each sample. In the case of bacterial dereplication, the variation between strains is sufficient enough to produce unique

protein fingerprints for each bacterial isolate, which can be provided by the instrument within a few minutes (Mellmann *et al*., 2008). Spectra typically show a large number of peaks between m/z 3,000 and 10,000, representing the "fingerprint" generated (Jackson, 2001). An excellent, in-depth summary of MALDI-TOF MS has been published by Gerwick and Dorrestein (Esquenazi *et al*., 2008; Esquenazi *et al*., 2009).

MALDI is an advanced technology compared to traditional dereplication techniques owing to its high sensitivity (Lopez-Legentil *et al*., 2005), simplicity (Vargha *et al*., 2006), ability to analyze high mass ranges (Liu *et al*., 2007), low sample requirement (Jackson, 2001), reproducibility (Dieckmann *et al*., 2005; Wunschel *et al*., 2005), automation (Dieckmann *et al*., 2005), and time-saving benefits (Mellmann *et al*., 2008; Risch *et al*., 2010). For example, clinical diagnostics using MALDI-TOF MS profiling have shown a decrease in the overall analysis cost to 22 – 32% of the comparable and conventional bacterial identification methods (Sedo *et al*., 2010). Furthermore, the identification of clinical isolates traditionally takes 24 – 48 hours, whereas MALDI-TOF MS profiling classifies clinical isolates within a few minutes (Risch *et al*., 2010). Resulting from its high degree of sensitivity, MALDI-TOF MS may also serve as an alternative to 16S rRNA gene sequencing (Bizzini *et al*., 2010), as MALDI-TOF MS provides finer resolution than 16S rDNA sequences in some environmental bacteria (Dieckmann *et al*., 2005; Vargha *et al*., 2006). In addition, clinical isolates are classified more accurately than 16S rRNA gene sequencing (Mellmann *et al*., 2008). However, bacterial growth conditions – including time of growth and media selection – have an effect on the characteristic spectral fingerprint; therefore, this factor must be taken into account when analyzing MALDI-TOF MS profiles (Jackson, 2001). This can be mitigated by maintaining consistencies with respect to growth time and media choice. Furthermore, the use of MALDI-TOF MS for the dereplication of

unknown environmental isolates is still in its infancy, with the first study describing its use to dereplicate marine environmental isolates published in 2005 (Dieckmann *et al*., 2005). As pointed out by this research group, phylogenetic assessment of unknown bacteria from unknown clusters in MALDI-TOF MS analyses will undoubtedly always rely on selective sequencing, notably by the use of the 16S rRNA gene (Dieckmann *et al*., 2005).

Previously, MALDI-TOF MS was applied to de-replicate sponge associated bacterial communities in four Norwegian boreal sponges (Dieckmann *et al*., 2005). Here, the authors employed MALDI-TOF MS on whole bacterial cells and discovered that MALDI resolved the strains into 11 groups corresponding to various species of Gammaproteobacteria, Firmicutes, and Bacteroidetes. This study found a large number of cultivable marine *Pseudoalteromonas* spp., which allowed for the examination of isolate differentiation at the species to strain level. Although virtually indistinguishable based on partial to full length 16S rRNA gene sequencing, the authors found that the isolated *Pseudoalteromonas* spp. could be readily distinguished via their MALDI-TOF MS patterns. This resolution was deemed significant for microbial drug discovery since closely related species may produce different compounds (Dieckmann *et al*., 2005). Hence, MALDI screening can provide valuable low level taxonomic resolution and is an excellent tool for dereplicating both clinical and environmental isolates.

NATURAL PRODUCTS

As Newman and Cragg (2004) eloquently stated, the lack of predation on sessile, soft-bodied plants and invertebrates piqued scientific curiosity: why were certain plants and invertebrates not attacked by organisms higher in the food chain? Why were some organisms susceptible to fouling, while others were not? It is widely agreed that these organisms produce compounds which deter potential consumers and spatial competitors by chemical defense

(Newman and Cragg, 2004). These compounds, called natural products or secondary metabolites, are compounds which are not essential to the sustenance of life yet offer an evolutionary advantage in their interactions with the environment (Bialonska and Zjawiony, 2009). To maintain their evolutionary selection, natural products presumably have increased the overall fitness of the host organism (Firn and Jones, 2009) owing to their high metabolic cost (Bialonska and Zjawiony, 2009). Isolated from higher plants, invertebrates, and microorganisms (Berdy, 2005), secondary metabolites have attracted biomedical interest for their wide range biological activities against human ailments, including antimicrobial, anticancer, antiviral, antiparasitic, and antiprotozoal activities (reviewed in Berrué and Kerr, 2009; Blunt *et al*., 2012; Cragg and Newman, 2005; Dias *et al*., 2012; Mayer *et al*., 2011). In their natural environment, biological functions of secondary metabolites are broad and appear to have roles in chemical defense (Gil-Turnes *et al*., 1989; Kelman *et al*., 2009), predation (Olivera *et al*., 1985), competition for space (Koh and Sweatman, 2000), protection from ultraviolet radiation (Thornton and Kerr, 2002), and anti-fouling (Lopez-Legentil *et al*., 2005). For example, bioassay guided fractionation revealed the compound 2,3,4 tribromopyrrole in the mollusc *Saccoglossus kowalevskii* was a chemical deterrent to fishes (Kicklighter *et al*., 2004). Another popular theory adopted by many chemical ecologists is that natural product diversity is the consequence of a "chemical arms race": the host gained an adaptive advantage by producing a bioactive molecule which could reduce the fitness of a competitor (Firn and Jones, 2003).

TERRESTRIAL NATURAL PRODUCTS

Although their ecological functions are often unknown, humans have benefited from the medicinal potential of secondary metabolites for thousands of years. The earliest suspected record in human medical history is based on Paleoanthropological studies of a 60,000 year old

Neanderthal burial site in northern Iraq (Solecki, 1975). Buried with the Neanderthals were eight different species of flowers, seven of which are recognized today for their herbal and medicinal properties. The flowers were not accidentally introduced into the grave – as demonstrated from the diverse habitat range from which the flowers were collected – rather, they must have been collected and purposely laid down with the burial. Hence, speculation rises as to whether or not the Neanderthals were aware of the medicinal values of the various plants used in the burial. Traditionally, higher plants have formed the basis of medicine, with an estimated 77% of plant derived drugs forming a basis in traditional medicine (Cragg and Newman, 2005) and today at least 119 chemical entities, derived from 90 plant species, are used as pharmaceuticals in one or more countries (Newman *et al*., 2000). However, only 5 – 15% of the ~250,000 species of higher plants have been assessed for biomedical potential (Cragg *et al*., 1999). As such, one might rationalize that a number of bioactive metabolites are awaiting discovery. The current challenge is finding new ways or new sources to access nature's chemical diversity (Harvey, 2000).

Economic success of terrestrial natural products. Not only are natural products and synthetic drugs based on natural products (termed "natural product derived drugs") successful at treating human ailments, they are also significant contributors to the profitability of many companies (Butler, 2004). Approximately one half of the 20 best selling drugs are either natural products or have been derived from structures provided by nature, with sales totaling roughly US$16 billion (Proksch *et al*., 2003; Tulp and Bohlin, 2002). Probably the most well-known example to date is acetylsalicylic acid (aspirin), an anti-inflammatory natural product derived from salicin found in the bark of the willow tree *Salix alba* L. (Dias *et al*., 2012), which also serves as inspiration for many other commonly used anti-inflammatory agents in use today. Aspirin alone generates a revenue of approximately US$ 8 billion for Bayer (Jennewein, 2005).

Augmentin®, an antibacterial, sold over US $1.5 billion worldwide in 1997, and the patent did not expire until 2002 (Newman *et al*., 2000). Finally, a lovastatin (cholesterol-lowering drug from a fungus) derivative, simvastatin (Zocor) had sales in excess of $4.3 billion in 2006 (before loss of patent protection) (Li and Vederas, 2009). Thus, natural product drug discovery is a profitable endeavor. Several examples below highlight the significance and success of natural products isolated from the terrestrial environment.

The "Golden Age" of Antibiotics. Discovered in 1928 by Dr. A. Fleming at St. Mary's Hospital in London, the observable lysis of pathogenic Gram-positive bacteria caused by penicillin began a revolutionary new path for antibiotic discovery and marketing. Although Dr. Fleming was not immediately aware of the significance of his own discovery until Florence and Chain published its biomedical potential in 1940 (Chain *et al*., 1940), the drug since went on to change health care for the better and led to the understanding of human infectious disease more than any other natural product to date (Rishton, 2008; van den Berg, 2008). Furthermore, penicillin represented the first drug which was powerfully active against many previously serious diseases, including syphilis, meningitis, and diptheria. As such, the "blossoming" of drug discovery followed after the large scale production of penicillin for WWII, which encouraged pharmaceutical industries to refocus their efforts on the search for new antibiotics (Baker *et al*., 2007; Dias *et al*., 2012). This marked the revolution of "the Golden Age of Antibiotics", which refers to the time period from the 1940s to the 1970s and whose name was derived by the sheer number of antibiotics discovered during this time (Davies, 2006). It was during this time (1941) that the word "antibiotic" was coined by Selman Waksman to describe any small molecule produced by a microorganism which inhibited the growth of other microorganisms (Davies, 2006). Antibiotics discovered in the Golden Age were mainly isolated from fungi and

actinomycetes (Pelaez, 2006), which represents one half of the FDA-approved antibiotics still in use today (Davies, 2006; Williams, 2009). Notably, a particular actinobacterial genus *Streptomyces* sp. represented 70 – 80% of all the isolated compounds during this time (Berdy, 2005), which were active against a wide range of bacterial and fungal infections (Berdy, 2005; Newman *et al*., 2000). The high number of microbial natural products discovered in the Golden Age of Antibiotics is not surprising, since some bacteria dedicate up to 20% of their genome to secondary metabolite biosynthesis (Yang *et al*., 2009). For example, 50 minor compounds were produced in addition to gentimicin in a *Micromonospora* sp. (Berdy *et al*., 1977). Owing to the success of microbe-derived products in treating serious human ailments, industries were inspired to invest time and resources into natural product drug discovery, particularly in the form of establishing microbiological and fermentation programs (Baker *et al*., 2007). Major antimicrobial classes isolated during the Golden Age and beyond are displayed in Figure 1.2.

Actinobacteria as prolific producers of bioactive metabolites. Actinobacteria represent a phylum of Gram-positive bacteria recognized for their high guanine-cytosine (GC) content (Hopwood, 2007; Stackebrandt *et al*., 1997). Actinomycetes account for approximately 7,000 compounds reported (Jensen *et al*., 2005) and have been the origin of a significant number of marketed antibiotics (Pelaez, 2006), accounting for more than half of the bioactive secondary metabolites isolated from bacteria so far (Lam, 2006). The genome sequencing project of the first marine actinomycete revealed an unprecedented devotion to secondary metabolite biosynthesis (Lane and Moore, 2011), and with a genome size of up to 8 megabases, the genomes of actinomycetes are much larger than what is necessary for the basic sustenance of life (Hopwood, 2007). As a result of its extensive genome size, it is widely believed that a significant portion of the genome may encode genes for secondary metabolite pathways (Hopwood, 2007). The

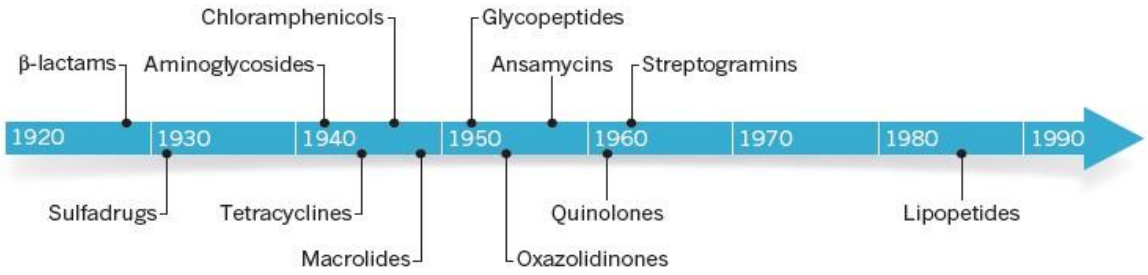


Figure 1.2: The timeline of major natural products isolated during the "Golden Age" and beyond (Lewis, 2012).

secondary metabolite potential of *Actinobacteria* is particularly illustrated by the genus *Streptomyces*, which thrive in complex environments such as soil and sediment. Their competitive advantage in these complex environments is attributed to their catabolic enzyme activity, which enables *Streptomyces* sp. to exploit complex polymers such as starch, cellulose, and chitin; the likes of which many other microorganisms cannot utilize (Hopwood, 2007; Williamson *et al*., 2000). Furthermore, *Streptomyces* sp. account for 80% of the actinomycete derived natural products reported to date, a value beyond rival in the microbial world (Jensen *et al*., 2005). For example, one metabolically talented *Streptomyces* sp. reportedly synthesizes at least 30 different secondary metabolites, many of which were new compounds at the time of description (Schiewe and Zeeck, 1999). Currently, an estimated 1 – 3% of all *Streptomyces* sp. antibiotics have been discovered (Clardy *et al*., 2006), implying the promising biomedical potential of natural products from this important group of bacteria is only beginning to be understood (Gulder and Moore, 2009; Jensen and Fenical, 1996). The remaining majority of as-of-yet undiscovered actinobacteria represent a large, untapped source of chemical diversity which may be useful in drug discovery programs (Manyak and Carlson, 1999).

DECLINE OF NATURAL PRODUCTS RESEARCH

As highlighted in the examples above, financially and medically successful drugs have been isolated from various terrestrial organisms, including higher plants and microorganisms. Although industries were considerably involved in finding medicinal cures for common human ailments from terrestrial sources in the Golden Age, pharmaceutical companies have since terminated or significantly reduced their natural product pipeline (Butler, 2004). For example, in the 1970s approximately 60 – 70 new chemical entities (NCEs) were introduced, which dropped

to 40 NCEs introduced in the mid-nineties (Manyak and Carlson, 1999). Multiple valid reasons can explain this decline. First, antibiotic research declined due to "solving" the perceived medical need, as the major causes of death caused by bacterial and fungal infections had seemingly been controlled by existing antimicrobials (Peláez, 2006). In addition, the cost of drug investment nearly tripled from US$10 billion to US$30 billion from the 1980s to the 2000s, while the return output of new drugs had considerably declined (Butler, 2004; Koehn and Carter, 2005). The lengthy delay – usually 10 years or more – between the discovery and market launch of a new drug onto the market also impacted the decision to invest in new medicines (Koehn and Carter, 2005). Finally, the terrestrial environment had all been seemingly exhausted as a source for new antimicrobial compounds (Davies, 2006).

Today, more than 30,000 diseases have been described, yet less than one-third can be treated and only a few cured (Zhang *et al*., 2005). However, industries are expected to provide safe/effective therapeutic agents (the humanitarian goal) and they also are expected to provide profitable drugs (the business goal; Baker *et al*., 2007). However, businesses cannot prosper and survive – and thereby cannot produce any drugs at all – if there is no financial return for their efforts in this market-based economy; hence, preference is given to those treatments which will offer considerable commercial success for their drugs (Baker *et al*., 2007). Consequently, short term cures, including antibiotics, have been grossly under investigated in comparison to drugs which are consumed daily for lengthy periods of time such as cholesterol lowering drugs (Baker *et al*., 2007; Li and Vederas, 2009).

Although preference is given to highly profitable drugs, of serious and immediate concern is the increasing crisis of new and re-emerging microbial infectious diseases to which no effective antimicrobials are available (Cragg, 1997). As the use of an antibiotic invariably selects

for resistant microbes, there is a continuous need for discovering new antibiotics (Clardy *et al*., 2006). For example, highly resistant strains of the "superbug" methicillin-resistant *Staphylococcus aureus* (MRSA) were originally restricted to hospitals, yet are now widespread across the country (Lewis, 2012). Additionally, resistance to the β-lactam class of antibiotics has rendered drugs such as penicillin less effective (Baker *et al*., 2007). From a public health standpoint, there is an urgent pressure for researchers to invest in new antimicrobial agents.

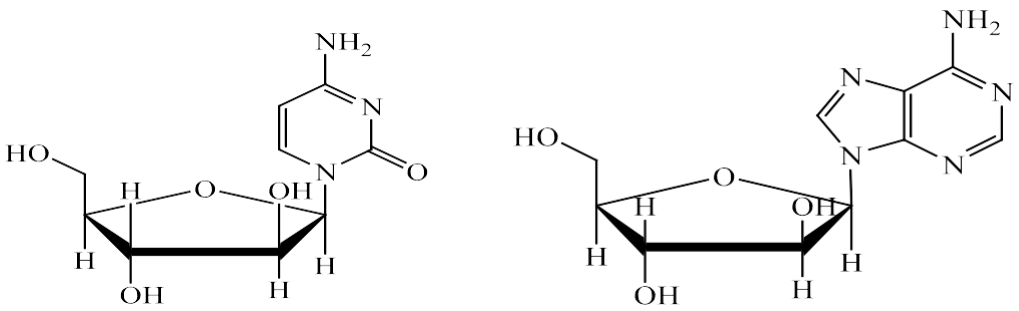
MARINE NATURAL PRODUCTS

With the need for new antibiotics and medical treatments on the rise, over-mining of the terrestrial environment resulted in diminishing returns and the drug discovery platform collapsed (Kim, 2012). With regard to drug discovery and development, hope was provided in that the oceans began attracting interest from pharmaceutical companies and research institutions following the advent of the self-contained underwater breathing apparatus (SCUBA) (1970s), manned submersibles (1980s), and remotely operated vehicles (ROVs) (1990s) (Dias *et al*., 2012). Now, organisms which were previously unavailable to researchers could be accessed with significant ease.

In terms of natural product discovery, the marine environment provides a rational ecosystem for identifying new bioactive compounds which may be distinct compared to their terrestrial counterparts. With over 300,000 described marine species representing 34 of the 36 described phyla (Donia and Hamann, 2003), the number of marine phyla surpasses that of the terrestrial environment (Sala and Knowlton, 2002), and represents the most biologically and chemically diverse habitat on the planet (Burgess, 2012). Furthermore, life began in the sea (Sala and Knowlton, 2002), and today the world's oceans cover over 70% of our planet's surface (Esquenazi *et al*., 2008). A wide variety of unique habitats are found within the world's oceans,

ranging from tropical, shallow water coral reefs to sub-zero, deep seas which harbour uniquely marine plant and invertebrate phyla (Jensen and Fenical, 1996).

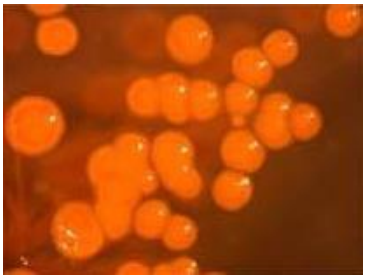
Despite offering a wide variety of unique habitats suitable for discovering new taxa, the oceans represent a relatively untapped yet rich resource for identifying bioactive natural products with applications in the pharmaceutical, cosmetic, agricultural, and nutritional supplements, each representing products with a potential multi-billion dollar market value (Ebada *et al*., 2008; Pomponi, 1999). The first marine natural products were identified in the 1950s by Bergman and Feeney, who isolated the unusual nucleosides spongothymidine and spongouridine from the Caribbean sponge *Cryptotethya crypta* (Bergman and Feeney, 1951). These structures served as lead structures for the discovery of the FDA approved drug for leukemia, Ara-C (**1**, Figure 1.3) and the anti-viral drug Ara-A (**2**). Since then, more than 20,000 structurally diverse marine natural products with unique bioactivities have been identified from marine microbes, algae, and invertebrates (Baker *et al*., 2007; Hu *et al*., 2011) representing a wide variety of natural product classes, including terpenes, acetogenins, alkaloids, and polyphenolics, with notable antifungal, antiviral, antibacterial, antiparasitic, anticancer, and antiprotozoal activities (reviewed in Blunt *et al*., 2012; Butler, 2008; Mayer *et al*., 2011; Newman *et al*., 2000). To date, the global marine pharmaceutical pipeline consists of four US-FDA approved marine-derived drugs (ziconotide, eribulin mesylate, cytarabine, vidarabine), one EU registered drug (ET-743), and 13 natural products which are in various phases of the clinical or pre-clinical pipeline (Mayer *et al*., 2010; Montaser and Luesch, 2011).



1 **2**

Figure 1.3: The sponge derived nucleosides Ara-C (**1**) and Ara-A (**2**).

As addressed by Jensen and Fenical (1996), it is with little wonder that marine natural products show little similarity to those isolated from the terrestrial environment, where completely different environmental challenges must be faced. Unlike their terrestrial counterparts, marine organisms must adapt to different environmental stressors including high pressure (up to ~1,100 atmospheres), salinity, limited light penetration, anaerobic environments at temperatures near freezing (0°C on the deep sea floor), high acidity (pH as low as 2.8), oligotrophic conditions, and temperatures exceeding 100°C near hydrothermal vents at the mid-ocean ridges (Lam, 2006). Furthermore, unlike their terrestrial counterparts, marine organisms must compensate for the enormous dilution factor owing to the aqueous nature of their environment which causes diffusion of their bioactive metabolites; consequently, marine natural products can be incredibly potent (Zhang *et al*., 2005). Marine organisms must therefore rely on unique adaptation strategies to deal with stress which may have resulted in the evolution of unique metabolic and physiological pathways (Jensen and Fenical, 1996), resulting in secondary metabolites which can differ significantly from those isolated in the terrestrial environment (Fenical, 1993). For instance, halogenation is a common phenomenon in marine natural products, presumably owing to the ready availability of chlorine in seawater (Lam, 2007; Simmons *et al*., 2005) and is vital for bioactivity in some marine secondary metabolites (Jensen *et al*., 2005). This is particularly highlighted by the marine metabolite Salinisporamide A, (**3**, Figure 1.4; Nereus Pharmaceuticals) a γ-lactam-β-lactone which displays potent cytotoxicity against HCT-116 human colon carcinoma (IC_{50} value of 11 ng/mL) (Williams *et al*., 2005). The growth of the producing actinomycete, *Salinispora tropica*, (Figure 1.4) is dependent on sodium concentration (Lam *et al*., 2006) and the loss of the chloroethyl moiety reduces biological activity by a 500-fold reduction as demonstrated previously (Williams *et al*., 2005). FDA approved drugs from



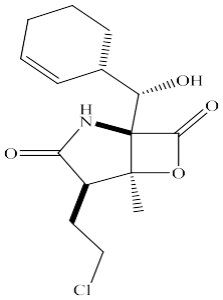


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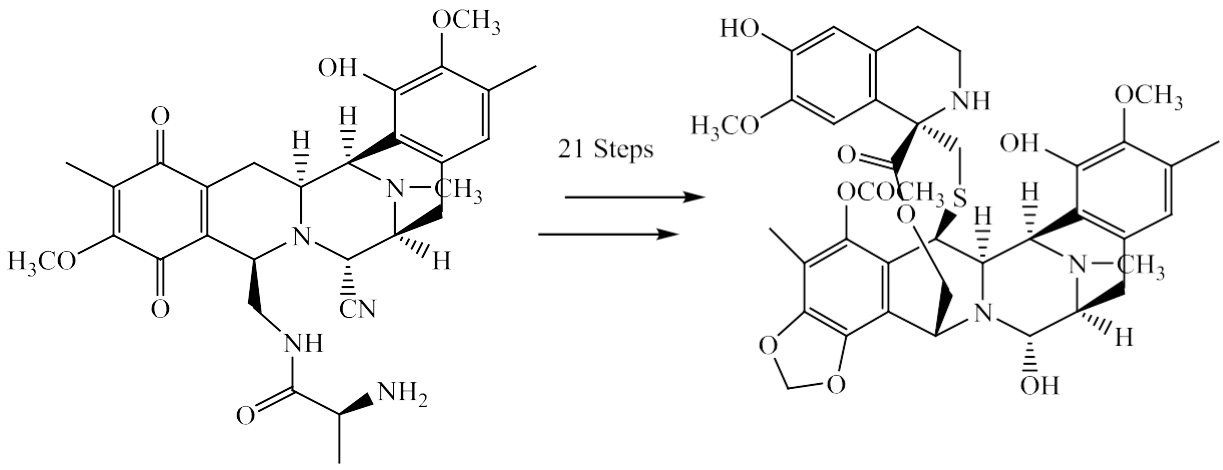
Figure 1.4: The Actinomycete *Salinispora tropica* and its halogenated natural product, Salinisporamide A (**3**). Image from Mayer *et al*., 2010.

marine sources are reviewed by Mayer (2010), and marine natural products with significant biomedical and financial value are discussed below and highlighted in Table 1.1.

ET-743. Undoubtedly one of the most successful marine natural products to date is ET-743 (Yondelis®, generic trade name trabectedin; PharmaMar/Johnson & Johnson), (**4**, Scheme 1.1) which was isolated from the tunicate *Ecteinascidia turbinata* (Figure 1.5) from mangroves in the Caribbean and Mediterranean Sea (Mayer *et al*., 2010; Rinehart *et al*., 1990; Zewail-Foote and Hurley, 1999). Its impressive *in vitro* nanomolar potency in preclinical trials against a wide variety of human cancer cell lines promoted its rapid progression to Phase I clinical trials in the 1990s (Izbicka *et al*., 1998; Rinehart *et al*., 1990; Zewail-Foote and Hurley, 1999).This compound was the first marine anticancer drug to be approved in the EU for patients with soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer (Mayer *et al*., 2010). As of 2002, it had been used to treat 1,000 patients in European and American hospitals (Proksh *et al*., 2002). However, its progression through clinical trials was delayed due to a low source of supply (Proksch *et al*., 2002). Although total synthesis had been published, the 44-step procedure resulted in a yield of less than 1% (Rinehart, 1999). However, researchers at PharmaMar reported a scientific breakthrough by performing a 21-step, semi-synthesis starting with cyanosafracin B (**5,** Scheme 1.1), an antibiotic of bacterial origin which can be produced in a cost-effective, multi-kilogram scale by bulk fermentations of the bacterium *Pseudomonas fluorescens* (Cuevas *et al*., 2000). Interestingly, this bacterial metabolite was a close analog of the ecteinascidin, and therefore provided a platform for the generation of this important anticancer agent.

Table 1.1: Marine natural products in the clinical pipeline. Adapted from Mayer *et al.* (2010) and Haefner (2003).

Compound Name	Clinical Status	Original Marine Source	Natural Product Class	Treatment	Molecular Target	Licensing Company	Reference
Ara-C (Cytosar-U®)	Approved	The sponge *Cryptotethya crypta*	Nucleoside	Cancer	DNA polymerase	Bedford, Enzon	Bergman and Feeney, 1950; Furth and Cohen, 1968.
Ara-A (Vira-A®)	Approved	The sponge *C. crypta*	Nucleoside	Antiviral	Viral DNA polymerase	King Pharmaceuticals	Bergman and Burke, 1955; Furth and Cohen, 1968
Ziconotide (Prialt®)	Approved (FDA; EU)	The cone snail *Conus magus*	Peptide	Analgesic	N-type Ca^{2+} channels	Elan Pharmaceuticals	Olivera and Cruz, 2001
ET-743 (Yondelis™)	Approved (EU only)	The tunicate *Ecteinoscidia turbinata*	Alkaloid	Cancer	Minor groove of DNA	Pharmamar	Rinehart *et al*., 1990; Izbicka *et al*., 1998; Rath *et al*., 2011
Eribulin Mesylate (Halaven™)	Approved (2010)	The sponge *Halichondria okadai* (derivative)	Macrolide	Cancer	Microtubules	Eisai Inc.	Stamos *et al*., 1997; Molinski *et al*., 2009 (reviewed)
DMXBA (GTS-21)	Phase II	The Nemertine worm *Paranemertes* sp.	Alkaloid	Alzheimers Disease; Schizophreni	Nicotinic acetylcholine receptor	Comentis; Taiho	Kem *et al*., 2006
Discodermolide	Phase I	The deep-water sponge *Discodermia dissoluta*	Polyketide	Cancer	Microtubules	Novartis	Gunasekera *et al*., 1991
Methopterosin (OAS1000)	Phase II complete	The octocoral *Pseudopterogorgia elisabethae* (Derivative)	Diterpene glycoside	Wound healing; inflammation	PLA_2	OsteoArthritis Sciences	Fenical, 1997
Pseudopterosins	Phase II complete	The octocoral *Pseudopterogorgia elisabethae*	Diterpene glycoside	Inflammation, analgesic, cosmetic applications	PLA_2	NA	Look *et al*., 1986a,b; Mayer *et al*., 1998
Salinosporamide A (Marizomib)	Phase I	The marine bacterium *Salinisporamide A*	β-lactone-γ-lactam	Cancer	20S proteasome	Nereus Pharmaceuticals	Williams *et al*., 2005
Bryostatin 1	Phase I complete	The bryozoan *Bugula neritina*	Polyketide	Cancer	Protein kinase inhibitor	GPC Biotech	Pettit *et al*., 1982
Dolastatin-10	Phase II complete	The sea slug *Dolabella auricularia*	Peptide	Cancer	Microtubules	NCI/Knoll	Haefner, 2003



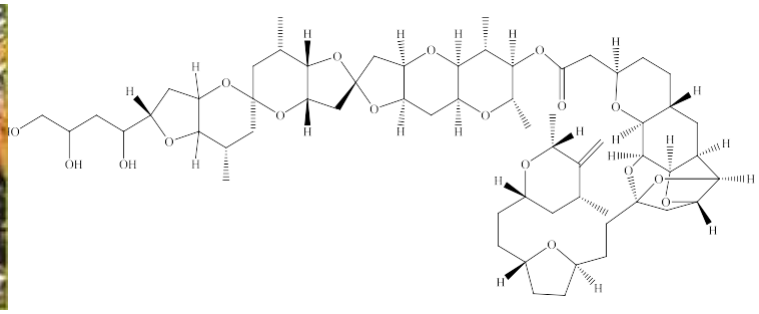
5 **4**

Scheme 1.1: The production of ET-743 (**4**) is generated semi-synthetically using cyanosafracin B (**5**), a metabolite of bacterial origin.

Figure 1.5: The sea squirt *Ecteinascidia turbinata*. Image obtained from Li and Vederas (2009).

Eribulin mesylate. A second example of a marine natural product with exceptional medical and commercial value is that of the FDA-approved halichondrin B (**6**, Figure 1.6) derivative eribulin mesylate (Halaven™, Eisai) (**7**). Since their discovery from the sponge *Halichondria okadai* (Figure 1.6) by researchers in Japan in the mid-1980s and subsequently in the deep water sponge *Lissodendoryx* sp. in the 1990s, the halichondrins have been the subject of numerous investigations owing to their potent *in vivo* antitumor activity (Hirata and Uemura, 1986). Owing to their phenomenal potency, even small quantities of Halichondrin B could be commercially valuable (Simmons *et al*., 2005) and as of 2002, the promising bioactivity of Halichondrin B encouraged the US National Cancer Institute to progress the compound to Phase I clinical trials (Molinski *et al*., 2009). However, the supply is extremely limited in the invertebrate host, yielding approximately 0.4 mg of halichondrin B per kilogram of *Lissodendoryx* sp. (Proksch *et al*., 2003). In order to be approved as an FDA drug for cancer treatments, an estimated one to five kg per year is required, requiring roughly 3,000 – 16,000 metric tonnes of sponge biomass annually (Proksch *et al*., 2003). Although two different sponge species contained Halichondrin B – which would thereby mitigate some ecological damage caused by overharvesting one species – the supply paradigm prevented full clinical use. Although total synthesis was reported in the early 1990s, the procedure took 90 steps to perform (Aicher *et al*., 1992a, b) and still resulted in poor yields.

Ingeniously performed by researchers in Japan, a significant breakthrough was achieved by truncating synthetic analogues of halichondrin B. Here, it was demonstrated that only the right-hand portion of the molecule was required for nearly equal activity with Halichondrin B against tumor cells (Stamos *et al*., 1997; Wang *et al*., 2000). The bioactivity was reported to reside in the macrocyclic lactone corresponding to the C1 – C38 moiety, and this derivative was



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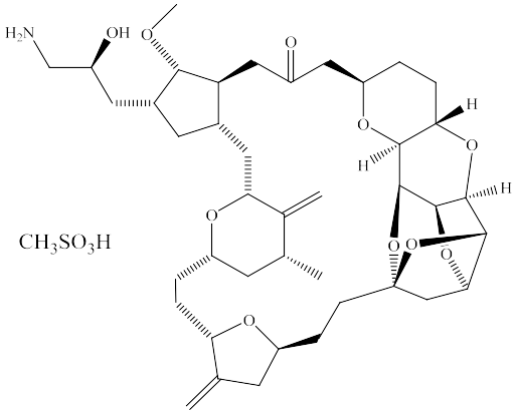

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Figure 1.6: The sponge *Halichondria okadai* (image obtained from Mayer *et al*., 2010), Halichondrin B (**6**) and Eribulin mesylate (**7**).

named eribulin mesylate (Stamos *et al*., 1997; Wang *et al*., 2000). This work culminated in the scale up of eribulin mesylate (Molinski *et al*., 2009), which could be generated in quantities suitable for clinical investigations (Huyck *et al*., 2011). Today, eribulin mesylate is approved by the European Union for use in patients with advanced breast cancer (Molinski *et al*., 2009), and is in various other clinical trials due to its *in vitro* activity against a variety of other cancer cell lines (McBride and Butler, 2012).

NATURAL PRODUCTS FROM CORALS

Natural Products from Scleractinians

As highlighted in the examples above, the marine environment offers a unique array of secondary metabolites which have significant biomedical applications. Scleractinian corals are the primary builders of coral reefs, yet are traditionally not known to produce secondary metabolites, presumably due to their physical protection via a calcareous exoskeleton (Coll, 1992). Notable exceptions of marine natural products isolated from scleractinians include alkaloids, primarily the aplysinopsins (Bialonska and Zjawiony, 2009; Koh and Sweatman, 2000), macrolides (Rashid *et al*., 1995), and cytotoxic sterols (Guerriero *et al*., 1996).

Natural Products from Octocorals

In contrast to scleractinian corals, chemical investigation of alcyonacean octocorals over the past several decades has uncovered a wealth of structurally diverse metabolites which possess an equally diverse array of biological activities. The identification of large quantities of prostaglandins (which have biomedical applications involved in inflammatory disease, fever, and pain) from the alcyonacean *Plexaura homomalla* by Weinheimer and Spraggins in 1969 has been

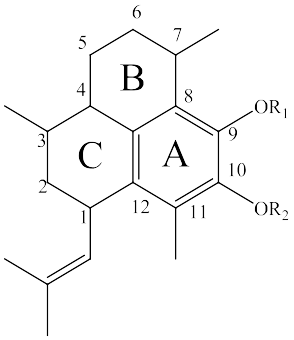
considered as the "take-off point" for serious efforts of drug discovery from the sea (Proksh *et al*., 2002). Since then, the common structural classes isolated from alcyonacean octocorals includes sesqui- and diterpenes (Berrué and Kerr, 2009) and highly functionalized steroids (Coll, 1992; Fenical, 1987). In particular, diterpenes isolated from octocorals have a range of biological activities which can be applied to areas of human health, including anti-cancer (most common), anti-inflammatory, antiplasmodial, antibacterial (generally against Gram-positive bacteria), antiviral, and antioxidants (Berrué and Kerr, 2009; Berrué *et al*., 2011a). As a result of the low internal source of supply, many octocoral derived natural products have not become FDA approved drugs. For example, the encrusting octocorals *Eleutherobia* sp. and *Erythropodium caribaeorum* are the sources of the cytotoxic diterpene eleutherobin, which shows a similar nanomolar potency (IC_{50} is 10 ± 15 nM) and mechanism of action as paclitaxel against a broad range of selective cancer cell lines (Hamel *et al*., 1999; Lindel, 1998). However, eleutherobin is available only in scarce amounts, comprising between 0.01 – 0.03% of the dry weight of *Eleutherobia* sp. (Lindel, 1998). The related diterpene glycoside desmethyleleutherobin demonstrates comparable anticancer activity to Taxol with an IC_{50} of 20 nM (Berrué *et al*., 2011a). Similarly, the fuscosides, diterpenes isolated from the alcyonacean *Eunicea fusca*, demonstrate superior anti-inflammatory activity compared with the industry standard indomethacin by inhibition of the enzyme 5-lipoxygenase (Berrué *et al*., 2011a). Again, the supply of these important diterpenes in their native environment is low and their progression through clinical trials has consequently been hampered.

Natural products from *Pseudopterogorgia elisabethae*

Particular attention on natural products isolated from alcyonaceans has been given to the octocoral *Pseudopterogorgia elisabethae*. Chemical investigation on this important octocoral

was initiated by Fenical and co-workers in the mid-1980s following the discovery of the pseudopterosins (Look *et al*., 1986a,b), diterpene glycosides with potent anti-inflammatory and analgesic properties comparable to the industry standard indomethicin. Studies have since revealed that *P. elisabethae* is host to a remarkably diverse array of compounds with over 78 compounds representing 20 distinct terpene skeletons being reported (Ata *et al*., 2004; Berrué and Kerr, 2009; Rodriguez and Shi, 2000). Not only is the chemical diversity particularly valuable, the reported biological activities are also incredibly diverse and display notable cytotoxic, antibacterial, anti-inflammatory, antiviral, anti-tuberculosis, and analgesic activities (reviewed in Berrué and Kerr, 2009). The natural products of *P. elisabethae* and its associated biological activities have been reviewed in detail elsewhere (Berrué *et al*., 2011a; Flachsmann *et al*., 2010; Rodriguez *et al*., 2004).

Pseudopterosins. The pseudopterosins (Figure 1.7, general structure, **8**) were the first natural products to be isolated from Bahamian collections of the octocoral *P. elisabethae*. These diterpene glycosides demonstrated significant anti-inflammatory and analgesic activity (Look *et al*., 1986a, b) which were significantly more potent than indomethicin in blocking topical inflammation ($K = 8.93 \times 10^{-4}$ M for pseudopterosin A and 40 mM for indomethicin) in mouse ear models and were potent inhibitors of phospholipase A_2 (IC_{50} = 3.0 µM *in vitro*) (Look *et al*., 1986a, b; Mayer *et al*., 1998), the key enzyme involved in the biosynthesis of inflammatory eicosanoid mediators (Proksch *et al*., 2002). The most biologically active pseudopterosin discovered at the time – pseudopterosin A (PsA, **9**) – also displayed analgesic potential several times more potent than indomethacin (ED_{50} of PsA is *ca.* 3.12 mg/kg versus *ca.* 10 mg/kg for



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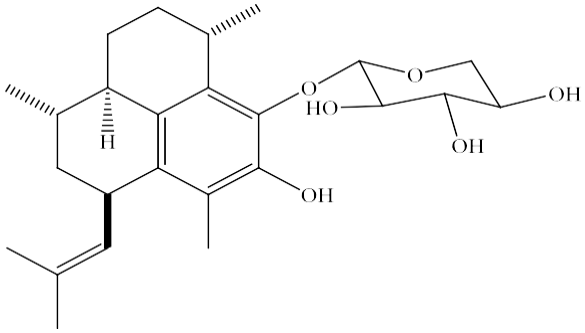

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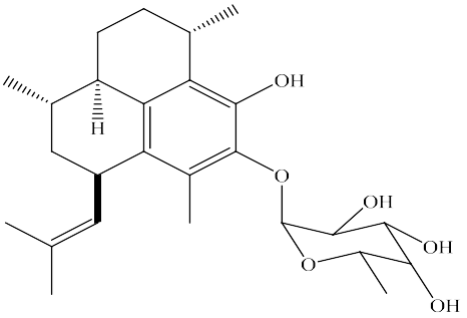

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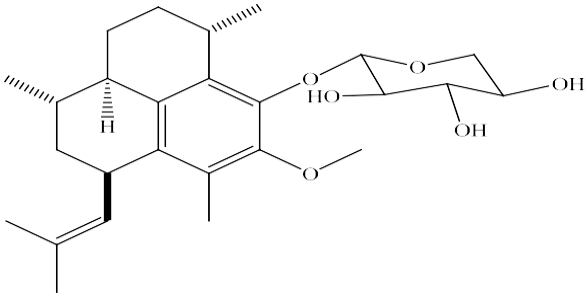

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Figure 1.7: The general structure of the pseudopterosins (**8**) and pseudopterosin analogues PsA (**9**), PsE (**10**), and methopterosin (**11**).

indomethacin) (Look *et al*., 1986a). Shortly thereafter, pseudopterosin E (PsE, **10**) was reported from a Bermudian collection of *P. elisabethae*, which was reportedly less acutely toxic in mice (LD_{50} > 300 mg/kg) than pseudopterosin A (Roussis *et al*., 1990). The pseudopterosins appear to act by a novel mechanism of action distinct from other non-steroidal anti-inflammatory drugs, which involves inhibiting eicosanoid biosynthesis in polymorphonuclear leukocytes (Kijoa and Sawangwong, 2004; Kohl and Kerr, 2004). A total of 31 pseudopterosins have been described which differ structurally in the location and identity of the sugar unit, yet the aglycone skeleton is identical (Berrué *et al*., 2011a). Today, the pseudopterosins are prized both as anti-irritants in cosmetic applications (Roussis *et al*., 1990) and for their progression past Phase II clinical trials as anti-inflammatory agents (Mayer *et al*., 2010). The pseudopterosins have also met with unexpected financial success for their use as additives in the Estée Lauder™ Resilience Lift line to prevent irritation caused by sun exposure (Kijjoa and Sawangwong, 2004) and for reducing skin degeneration (Fenical, 1997). Additionally, a simple derivative of the pseudopterosins, methopterosin (**11**), has been approved by the FDA for clinical trials (Correa *et al*., 2009) as a superior wound healing agent, which enhances wound healing by over 400% and is therefore an attractive treatment for burn victims and for disorders involved in the healing process (Fenical, 1997). However, as the coral is the sole known source of these biomedically important diterpenes, an estimated 30 – 45 tons of *P. elisabethae* have been harvested per year from the Bahamas since the mid-1990s (Gutierrez-Rodriguez and Lasker, 2004; Puyana *et al*., 2004). Hence, defining alternative and viable sources for generating suitable supplies is critical (Mydlarz *et al*., 2003). While the pseudopterosins have successfully completed Phase II clinical trials as a topical anti-inflammatory agent, further progression of the drug has been hindered by supply issues (Berrué *et al*., 2011a). Owing to the complexity of the structure and based on the

fact that the pharmacophore is not yet entirely understood (Berrué *et al*., 2011a), chemical synthesis is not an option; therefore, the pseudopterosins are awaiting further clinical testing until a suitable supply can be generated.

NATURAL PRODUCTS FROM MARINE BACTERIA

Microorganisms, particularly bacteria and fungi, produce the majority of all described clinically useful microbial natural products to date (Baker *et al*., 2007). Within the kingdom Bacteria, the majority of natural products isolated from marine bacteria between 1997 to 2008 producing all known anti-infective agents parallels the same five bacterial phyla as those described from the terrestrial environment: Actinobacteria (n = 256 compounds), Cyanobacteria (n = 220), Proteobacteria (n = 78), Firmicutes (n = 35), and Bacteriodetes (n = 34) (Williams, 2009). The Antibase database (a collection of all published microbial natural products) contains approximately 38,843 natural products, with about 1,000 derived specifically from marine microbes as of 2005 (Singh and Pelaez, 2008). Currently, the metabolite Salinisporamide A produced by the obligate marine bacteria *Salinispora tropica* is in clinical trials for the treatment of cancer (Mayer *et al*., 2010).

AN ISSUE CONCERNING MARINE NATURAL PRODUCT DRUG DISCOVERY

The supply issue. As has been identified above, a serious impediment causing delays in many marine natural products currently undergoing clinical or pre-clinical trials is the problem of low compound availability. In order to qualify as a drug, however, an adequate supply of the metabolite of interest must first be procured (Cragg *et al*., 1999). Nevertheless, yields of 1 mg of

the target compound per 3 kg of organism are not uncommon in many marine invertebrates (Newman and Cragg, 2004), often requiring collections of 5 – 50 kg of the host organism (Cragg *et al*., 1999; Proksch *et al*., 2002). It is easily apparent that invertebrate harvesting to supply the biologically promising natural product from nature cannot be performed without risking species extinction (Proksch *et al*., 2002). To mitigate the supply issue and to bridge the gap between medical potential and commercial application, numerous methods are generally considered.

MITIGATING THE SUPPLY ISSUE

Mariculture. Mariculture of the invertebrate, where the host organism is "farmed" in controlled areas, has been tested in some cases. For instance, bryostatin-1 and ET-743 can be generated at least partially by mariculture of the bryozoan *Bugula neritina* and the tunicate *E. turbinata* respectively (Mendola, 2000; Proksch *et al*., 2002). Prior to mariculture, bryostatins were collected by invertebrate harvesting off the southern coast of California where the invertebrate is most abundant, yet it took divers employed by the NCI two years to collect 17 tons of the organism (Haefner, 2003). However, weather events, pests, and infection can often render cultured marine invertebrates vulnerable to destruction (Fortman and Sherman, 2005). Furthermore, in most cases marine aquaculture still fails to produce sufficient yields of the metabolite of interest as required should the drug enter the market (Donia and Haman, 2003; Fortman and Sherman, 2005; Proksch *et al*., 2002). For example, the octocoral derived metabolite eleutherobin can be produced by mariculture of *Erythropodium caribaeorum*, which yields 0.0012% (wet weight), a value which cannot sustain clinical trials (Fortman and Sherman, 2005). Hence, other avenues of generating sufficient quantities of bioactive metabolites are needed.

Chemical synthesis. It is noteworthy to mention that natural product chemical structures can be extremely complex, costly to synthesize, and can still result in small yields (Baker *et al*., 2007; Fortman and Shermann, 2005; Proksch *et al*., 2003); thus, chemical synthesis is not always a viable option. Highly complex molecular structures are incredibly difficult to synthesize, and therefore, synthesis is not a practical means to commercially supply natural products. For example, the microtubule-stabilizing polyketide (+)-Discodermolide was coveted for its improved aqueous solubility (Koehn and Carter, 2005) and improved potency compared to paclitaxel (ter Haar *et al*., 1996), qualifying the compound as an exceptional anticancer candidate (Koehn and Carter, 2005). As is the case with many other marine invertebrates, discodermolide is isolated in low quantities in the host organism (0.002% wet weight) (Fortman and Sherman, 2005). Although its daunting structure was finally synthesized by the team at Novartis, the yields were still less than 1% (reviewed in Fortman and Sherman, 2005).

Heterologous host expression. A third approach to overcoming the supply issue is through heterologous host expression. As mentioned previously in this chapter, an estimated <1% of microbes are available in pure culture, yet cultivated marine microorganisms have yielded a number of promising therapeutics (Williams, 2009). In heterologous host expression, natural product biosynthetic genes are expressed in a microbial host which is amenable to culturing and production on a large scale (Clardy *et al*., 2006). By using genetic expression of uncultivated bacterial DNA in an easily cultured heterologous host, one can bypass cultivation by directly accessing the genes encoding for secondary metabolites (Brady *et al*., 2001; Clardy *et al*., 2006). The key to successful application lies in selecting a suitable host, which if chosen appropriately, should have a proper metabolic and genetic background in a close approximation to the native cellular environment (Clardy *et al*., 2006; Fortman and Sherman, 2005). The first

example of successfully cloning a marine natural product biosynthetic gene cluster from a bacterial symbiont was performed by Schmidt *et al*. (2005). Here, the authors reported the successful expression the peptides petallamide A and C into the heterologous host, *Escherichia coli*, revealing the practicality of heterologous host expression. Clearly, heterologous host expression has important implications for the production of otherwise inaccessible marine drugs. However, identifying natural product gene clusters from clone libraries from the presumed metabolic producer can be tedious and time consuming (Gulder and Moore, 2009), while differences in background metabolism can be challenging barriers rendering genes incompatible with the most commonly used hosts (Clardy *et al*., 2006).

Fermentation. A final method to overcome the problems of supply and sustainability of marine derived natural products is through fermentation. Fermentation can be a more cost effective alternative compared to chemical synthesis, as copious amounts of starting natural product material can be obtained from microbes which are amenable to culturing (Baker *et al*., 2007; Koehn and Carter, 2005). Furthermore, cultures can remain viable for many years without appearing to undergo degenerative changes (Backus and Stauffer, 1955), and can therefore generate a continuous supply using the same starting material. Microbial recovery is simple, requiring only aseptic removal of a small quantity of stock on a selected medium (Backus and Stauffer, 1955). Furthermore, natural product production via fermentation is rapid, and biosynthesis has been documented after only 22 hours of growth (Yang *et al*., 2009). Examples of unmodified marine secondary metabolites produced by bacterial fermentation include thiocoraline, salinosporamide A, and curacin A (Fortman and Sherman, 2005). However, it is also important to recognize that fermentation of a microbial natural product is only possible

when the metabolite of interest can be isolated from a viable microbial culture (Fortman and Sherman, 2005).

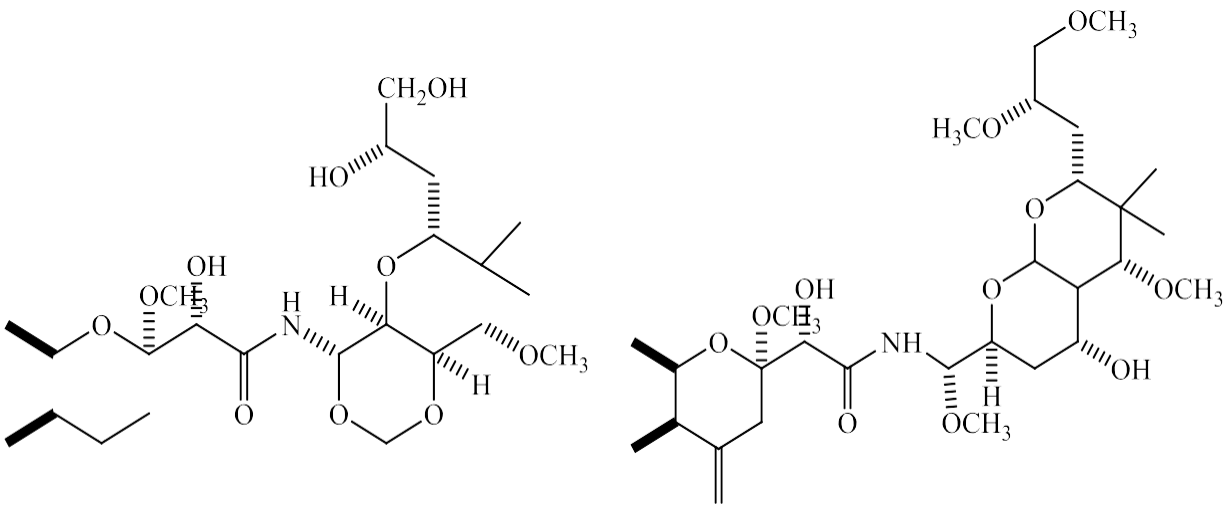
SYMBIOTIC MICROORGANISMS AS POTENTIAL BIOSYNTHETIC PRODUCERS OF NATURAL PRODUCTS

It is important to note that the presence of a secondary metabolite within a particular cell type is not indicative of its biosynthetic origin (Simmons *et al*., 2008). Although localization of natural products to a specific cell type may form the basis for some arguments regarding biosynthetic origin, in truth, localization does not necessarily correspond to the site of production (Fortman and Sherman, 2005). For instance, although a wide variety of biomedically important natural products have been isolated from invertebrates, recent theories based on genetic and radiolabeling studies have demonstrated that at least some natural products may be produced by their associated microbial endosymbionts (Boehnlein *et al*., 2005; Davidson *et al*., 2001; reviewed in König *et al*., 2006; Mydlarz *et al*., 2003; Schmidt *et al*., 2005; Sharp *et al*., 2007). As marine invertebrates harbor abundant populations of symbiotic microorganisms (Gulder and Moore, 2009) and given that many marine invertebrates have existed for hundreds of millions of years, it is reasonable to propose that symbiotic bacteria have co-evolved with the invertebrate host (Fortman and Sherman, 2005). Hence, it is indeed possible that the host invertebrate may be sequestering microbial-produced metabolites. However, such a relationship has yet to be conclusively proven (Fortman and Sherman, 2005), as bacterial endosymbionts are highly fastidious and have thus far avoided cultivation attempts (Piel *et al*., 2004). Other examples of symbiotic bacteria as possible producers of invertebrate-derived natural products have been reviewed elsewhere (König *et al*., 2006; Proksch *et al*., 2002) and are summarized in Table 1.2.

Table 1.2: Selected marine natural products from invertebrates suspected to be of microbial origin.

Compound	Host Invertebrate	Microbial Producer/Chemical Similarity	Reference
Taxol	The Yew *Taxus* sp.	The fungus *Seimatoantlerium tepuiense*	Tulp and Bohlin, 2002; Strobel *et al*., 1999
Mycalamides (including mycalamides, onnamides, and theopederins)	The sponge *Mycale; Theonella* sp.; and *Discodermia* sp.; the ascidian *Polysincraton* sp.	Similar to pederin (see below); suggestive of microbial origin	Dyshlovoy *et al*., 2012
Pederin	The beetle *Paederus fuscipes*	Uncultured *Pseudomonas aeruginosa* (bacterium)	Piel, 2002; Piel *et al*., 2004; Kellner *et al*., 2001, 2002
Swinholide A	The sponge *Theonella swinhoei*	*Candidatus Entotheonella palauensis* (bacterium); Cyanobacterial symbiont	Schmidt *et al*., 2005; Andrianasolo *et al*., 2005
Bryostatin-1	Bryozoan *Bugula neritina*	*Candidatus Endobugula neritina*	Davidson *et al*., 2001; Sudek *et al*., 2007; Sharp *et al*., 2007
ET-743 (Yondelis)	Tunicate *Ecteinoscidia turbinata*	*Pseudomonas fluorescens* (bacterium); Safracin B	Ikeda *et al*., 1983; Cuevas *et al*., 2000
Pseudopterosin	The octocoral *Pseudopterogorgia elisabethae*	The dinoflagellate *Symbiodinium* sp.; the bacterium *Pseudomonas aeruginosa*	Coleman and Kerr, 2000; Mydlarz *et al*., 2003; Newberger *et al*., 2006; Bunyajetpong *et al*., 2011
Jasplakinolide	*Jaspis* spp. (sponges)	*Chondromyces crocatus* (myxobacterium); Chondramide D	Proksch *et al*., 2003
Dolastatin 10	The mollusc *Dolabella auricularia*	*Symploca hydnoides* (cyanobacterium); Symplostatin-1	Luesch *et al*., 2001
Okadaic acid	The sponge *Halichondria* sp.	*Prorocentrum* sp. (dinoflagellate)	Proksch *et al*., 2003
Patellamides A and C	Ascidian *Lissoclinum patella*	Cyanobacterial symbiont	Schmidt *et al*., 2005
Aplysinamisin-1	The sponge *Aplysina aerophoba*	*Chloroflexi* phylotype (same compound)	Sacristán-Soriano *et al*., 2011

Pederin. One such means through which natural products are linked to microbial origin is through genetic analysis. For example, pederin (**12**, Figure 1.8) is a defensive polyketide isolated from female (+) beetles of the species *Paederus fuscipes* (Kellner *et al*., 2001), where the metabolite shows strong cytotoxicity against a panel of human cancer cell lines *in vitro* and *in vivo* (Proksch *et al*., 2003). However, 16S rRNA gene sequencing analysis of (+)-pederin containing (female) beetles revealed an endosymbiotic strain of the Gammaproteobacteria *Pseudomonas aeruginosa* was implicated as the most likely producer of the defensive polyketide (Kellner *et al*., 2001, 2002; Piel *et al*., 2002a, b). Cloning of the putative pederin biosynthetic gene cluster has been reported from the total DNA of *P. fuscipes* beetles (Piel *et al*., 2004). Sequence analysis of the *ped* gene cluster included a typical bacterial architecture consisting of a mixed modular polyketide synthase/nonribosomal peptide synthase and tailoring enzymes, thereby providing strong evidence of the prokaryotic origin of pederin (Piel, 2002). Using a similar approach, the authors discovered the structurally related compound, onnamide A, from the metagenome of the sponge *Theonella swinhoei*, to which putative gene clusters involved in natural product biosynthesis was linked to bacterial origin from an as-of-yet uncultivated bacteria (Piel *et al*., 2004). Furthermore, pederin shows a strong structural resemblance to the sponge derived cytotoxic metabolite mycalamide A (Figure 1.8, **13**), which suggests the latter metabolite may also be of bacterial origin (Proksch *et al*., 2003). This is due to the fact that it is generally accepted that isolating similar compounds from disperate regions of the world (including chemical conservation between the terrestrial and the marine environment), is indicative of a common microbial biosynthetic origin (Proksch *et al*., 2003). Conclusive experimental evidence for the hypothesis that microbial endosymbionts are the true biosynthetic producers has not yet been proven, as attempts to isolate the endosymbionts possibly responsible have thus far



12 **13**

Figure 1.8: The structural resemblance of mycalamide A (**25**) and pederin (**26**).

resisted cultivation on standard Zobell media (Proksh *et al*., 2003).

ET-743. Additional evidence supporting the hypothesis that many natural products originate from symbiotic (*sensu lato*) microorganisms is reflected by evidence from the FDA-approved anticancer agent ET-743. As described earlier, ET-743 (**4**) is isolated in low abundance from its native tunicate source *E. turbinata* (Zewail-Foote and Hurley, 1999). The chemical similarity of ET-743 to the *Pseudomonas fluorescens* (bacteria) originating metabolites the safracins is so pronounced that commercial production of ET-743 is produced partially synthetically using a biotechnologically available cyanosafracin B (Scheme 1.1, **5**, Cuevas *et al*., 2000; Ikeda *et al*., 1983). Naturally, one would assume, given that the microbe is the true producer, that the biosynthetic microbial producer would be persistently associated with the invertebrate host (Perez-Matos *et al*., 2007). One particular endosymbiont in *E. turbinata*, *Candidatus Endoecteinascidia frumentensis*, was found via molecular techniques to be specifically associated with *E. turbinata* from the Caribbean and Mediterranean, locations where the tunicate harbours ET-743 (Mendola, 2000; Perez-Matos *et al*., 2007). Furthermore, rigorous sequence analysis based on codon usage performed by Rath *et al*. (2011) suggested that the uncultivated bacterium *Candidatus E. frumentensis* contains the relevant biosynthetic machinery to produce ET-743, and the predicted enzyme activity for the tetrahydroisoquinoline core assembly was verified *in vitro*. Hence, based on the examples demonstrated herein and as pointed out by Proksch *et al*. (2002), it is tempting to assume that compounds such as ET-743 are introduced to their respective host invertebrate from their symbiotic microbial counterparts, rather than reflecting a mere chemical coincidence (Proksch *et al*., 2002).

Pseudopterosins. As mentioned previously, the octocoral *Pseudopterogorgia elisabethae* is the sole known source of the commercially and medically important compounds the

pseudopterosins. Initially assumed to be of invertebrate origin (Look *et al*., 1986a, b), convincing evidence from the Kerr lab suggests the true biosynthetic source of the pseudopterosins may be of microbial origin (Boehnlein *et al*., Kerr, 2005; Bunyajetpong *et al*., 2011; Bunjayetpong, 2011; Frenz-Ross *et al*., 2008; Mukherjee, 2003; Mydlarz *et al*., 2003; Newberger *et al*., 2006; Thornton and Kerr, 2002). For example, radiolabeling studies using ^{14}C-labeled inorganic carbon and the tritium labeled intermediate geranylgeranyl diphosphate (GGPP) revealed purified dinoflagellate symbionts free of *P. elisabethae* cells were capable of inducing pseudopterosin biosynthesis (Mydlarz *et al*., 2003). Additionally, a positive correlation between the number of *Symbiodinium* sp. cells and pseudopterosin concentration was observed. Finally, algal cells maintained in the dark did not produce ^{14}C-labeled pseudopterosins while algal cells maintained on a dark/light cycle produced pseudopterosins, providing strong evidence that the photosymbiotic symbiont was necessary for pseudopterosin biosynthesis. For the first time, these results revealed that pseudopterosins were produced independent of the host coral, yet the authors noted that zooxanthellae cells were not free of bacteria or fungi. As pseudopterosin biosynthesis was inhibited by 100 μM of mevastatin, a known anti-bacterial, this indicated pseudopterosin biosynthesis may involve prokaryotic biosynthetic machinery. This is particularly supported by the fact that pseudopterosins are present in the gametes of *P. elisabethae*, which themselves are free of symbiotic dinoflagellates (Mukherjee, 2003), while a pure *Pseudomonas aeruginosa* strain Ps137 subsequently was capable of producing pseudopterosins (Bunyajetpong *et al*., 2011). Hence, preliminary evidence suggests a bacterium may be the true producer of the pseudopterosins, yet further experiments are required to confirm that *P. aeruginosa* is truly responsible for pseudopterosin production.

CORAL-ASSOCIATED BACTERIA AS A UNIQUE NICHE TO INVESTIGATE NOVEL NATURAL PRODUCTS

As mentioned previously, the majority of species cannot be cultivated using today's technology. However, recent pyrosequencing analysis of the 16S rRNA gene sequence community of the Sargasso Sea demonstrated that the majority of phylotypes belonged to the "rare biosphere." If previously uncultured microorganisms dominate the total community, then this represents a significant, novel, and untapped resource for natural product drug discovery (Rondon *et al*., 1999). Interestingly, the previously described "rare biosphere", referring to the rare phylotypes uncovered by next generation sequencing, was sampled from marine water (Sogin *et al*., 2006). As coral associated bacterial concentrations on corals are higher than that of the surrounding seawater (Herndl and Velimirov, 1986), corals may prove to yield an even higher source of rare phylotypes. In drug discovery programs, maximum biological diversity is essential for discovering unique, novel chemical diversity (Singh and Pelaez, 2008). As corals harbor high levels of biological diversity via their associated bacteria, and given the hypothesis that a high biological diversity translates to a high chemical diversity, coral-associated bacteria may offer new natural products with unprecedented structural diversity. Interestingly, recent compelling evidence has suggested many of the approved drugs or drug leads previously assumed to be of invertebrate origin may be produced by their microbial inhabitants (Boehnlein *et al*., 2005; Davidson *et al*., 2001; Kador *et al*., 2011; König *et al*., 2006; Mydlarz *et al*., 2003; Sharp *et al*., 2007).

Although the scientific understanding of the ecological roles of marine natural products in octocorals is limited, the enormous benefit to the health and quality of life to mankind has maintained a continuous interest in their discovery and use; furthermore, characterizing the

healthy microfauna associated with these important marine invertebrates has general implications for reef conservation and management. Not only do coral extracts provide society with cures for disease and ailments, they also generates substantial revenue due to the scale-up and sale of bioactive compounds in an ecologically sustainable and continuous source of supply. In summary, as a result of their global prevalence and valuable economic, humanitarian, and ecologic benefits, the study of corals – including their microbial associates – is both a noble and worthy pursuit.

RESEARCH GOALS, HYPOTHESES, AND SIGNIFICANCE

Despite the growing wealth of knowledge regarding scleractinian-associated bacterial diversity, little is known regarding bacterial populations in alcyonacean octocorals. In addition, the culture-independent methodologies which have enhanced our knowledge regarding scleractinian coral-associated bacterial communities have only rarely been applied to studying bacterial communities residing within the octocoral holobiont. Not only would understanding bacterial communities in octocorals provide a basis for octocoral ecological management, but an exploration of their associated bacterial communities can also provide a resource for identifying novel secondary metabolites. Such discoveries will contribute to our understanding of octocoral associated bacterial communities and provide a resource for natural product discovery.

No study has investigated the bacterial diversity and natural product potential of bacteria associated with the octocoral *P. elisabethae.* This is a model organism to investigate octocoral-associated bacteria owing to the coral's broad geographic distribution and commercial significance (Berrué *et al*., 2011a). The thesis research goals herein are threefold:

1) To characterize the culture-independent bacterial community associated with *P. elisabethae,*

2) To determine the cultivable bacterial community associated with *P. elisabethae,* and

3) To assess the potential of cultivable bacteria to produce bioactive natural products.

Based on findings from scleractinian associated bacteria, this thesis hypothesizes that the bacterial community of *P. elisabethae* will be conserved both within and throughout reefs in the Bahamas, yet will be distinct from the bacterial community of the surrounding seawater. Due to the unique microhabitat offered by the octocoral *P. elisabethae,* it is hypothesized that microbial

symbionts capable of producing secondary metabolites with unique biological activities will be identified.

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CHAPTER II

A CULTURE-INDEPENDENT COMMUNITY AND DIVERSITY ANALYSIS OF BACTERIA ASSOCIATED WITH *PSEUDOPTEROGORGIA ELISABETHAE*

INTRODUCTION

Both stony corals and octocorals associate with a diverse collection of microorganisms, including algae, bacteria, and fungi, where many of these relationships are hypothesized to be mutualistic associations (Gray *et al*., 2011; Goulet and Coffroth, 2004; Rosenberg *et al*., 2007; Wang *et al*., 2011). Of these, prokaryotes appear to represent one of the richest sources of biological diversity within the host (Wegley *et al*., 2007). Coral-associated bacteria facilitate ecological processes, including nitrogen fixation (Lesser *et al*., 2004), the degradation of chitin and aromatic compounds (Rosenberg *et al*., 2007; Wegley *et al*., 2007), and aid in defense via the production of antimicrobial natural products (Nissimov *et al*., 2009). Despite the growing implications of the importance of such associations, microbiologists have long been restricted in their abilities to describe the composition of bacterial communities using traditional plating methodologies, a phenomenon known as "the great plate count anomaly" (Staley and Konopka, 1985). This phenomenon states that less than one percent of bacteria present in environmental samples can be cultured on standard agar plates (Amann *et al*., 1995).

With the discovery and utilization of the 16S rRNA gene for identifying microbes and determining phylogenetic relationships, culture-independent techniques have revolutionized our understanding of microbiology. Specifically, with the advent of bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) (Jonasson *et al*., 2002), rare bacterial phylotypes can be identified with superior sequencing depth in a cost-effective manner compared to traditional

methods (Amend *et al*., 2010; Sogin *et al*., 2006; Sunagawa *et al*., 2010). Investigations of coral associated bacteria using 16S rRNA gene sequencing began in 2001 by Rohwer and colleagues, and since then numerous publications have revealed scleractinian corals are host to a diverse array of associated bacteria (Bourne and Munn, 2005; Nithyanand and Pandian, 2009; Rohwer *et al*., 2001, 2002). In contrast, despite the wealth of information available regarding scleractinian-associated bacterial communities, very few investigations have examined bacterial diversity in healthy octocorals. The need to understand bacterial communities in healthy octocorals has largely been motivated by the notion that disease, coral bleaching, and death can occur when the natural microbial fauna is affected by opportunistic bacterial pathogens (Ben-Haim *et al*., 2003; Kushmaro *et al*., 2001).

The Caribbean octocoral *Pseudopterogorgia elisabethae* is the sole source of the commercially valuable pseudopterosins, diterpene glycosides which have potent anti-inflammatory activity and are marketed in the Estée Lauder® Resilience Lift cosmetic line (Fenical, 1997; Look *et al*., 1986). This organism was selected as a model to investigate octocoral-associated bacteria owing to its commercial value and the recent link suggesting the pseudopterosins may be of bacterial origin (Bunyajetpong *et al*., 2011; Bunyajetpong, 2011). Despite the high level of interest in *P. elisabethae*, no study has characterized the bacterial diversity associated with this octocoral from the Bahamas. The objective of this study was to characterize the bacterial microbiome of *P. elisabethae* and to determine whether the bacterial communities are conserved within and between-reefs throughout the Bahamas. Based on research suggesting bacteria form species-specific relationships with stony corals (Frias-Lopez *et al*., 2003; Koren and Rosenberg, 2006; Rohwer *et al*., 2001, 2002), this thesis tested the

hypothesis that the bacterial community in *P. elisabethae* will be conserved within and between reefs in the Bahamas.

MATERIALS AND METHODS

Coral sampling and collection information

Fragments of *Pseudopterogorgia elisabethae* corals (n=15) were collected by SCUBA from various locations throughout the Bahamas (Figure 2.1; 2006 collections: Eleuthera Island (site 1, n = 3; 24 48.55N, 76 20.58W); Sweetings Cay (site 2, n = 2; 26 36.23N, 77 54.70W), Victory Reef (site 3, n = 3, 25 29.13N, 79 16.41W), Petersons Reef (site 4, n= 3; 26 32.93N, 78 30.99W); all preceding specimens were collected by Dr. N. Ibrahim; 2009 collection: Victory reef, site 3, n = 1; 25 29.13N, 79 16.41W); collected by V. Robertson and R. Kerr. All corals appeared healthy at the time of collection ("healthy" as defined by the absence of coral bleaching and obvious tissue lesions) and were collected > 10 m apart. Prior to DNA extraction, all corals were washed by gently shaking three times with 50 mL of sterile filtered (0.22 µm) seawater to remove loosely associated bacteria. Following collection, corals were immediately frozen on dry ice and stored at -80°C until DNA was extracted. Water samples (500 mL, n = 2, W1 and W2, Figure 2.1) were collected in Ziploc bags surrounding site 3 in 2009 and were filtered through a 0.22 µm Nalgene MF575 water filtration device (VWR International, Mississauga, ON). The filter was aseptically removed from the filtration device and frozen on dry ice and stored at -80ºC until DNA isolation was performed. Microbes were collected from the filter membrane and purified using the Ultra Clean Water DNA kit (Mol Bio Laboratories, Carlsbad, CA) by R. Pike. Surrounding water was collected from site 3 (2009; Figure 2.1) and was not collected in 2006; thus, coral and water bacterial communities are defined herein as "similar" and "not similar" based on comparison of site 3, 2009-collected *P. elisabethae* to water.

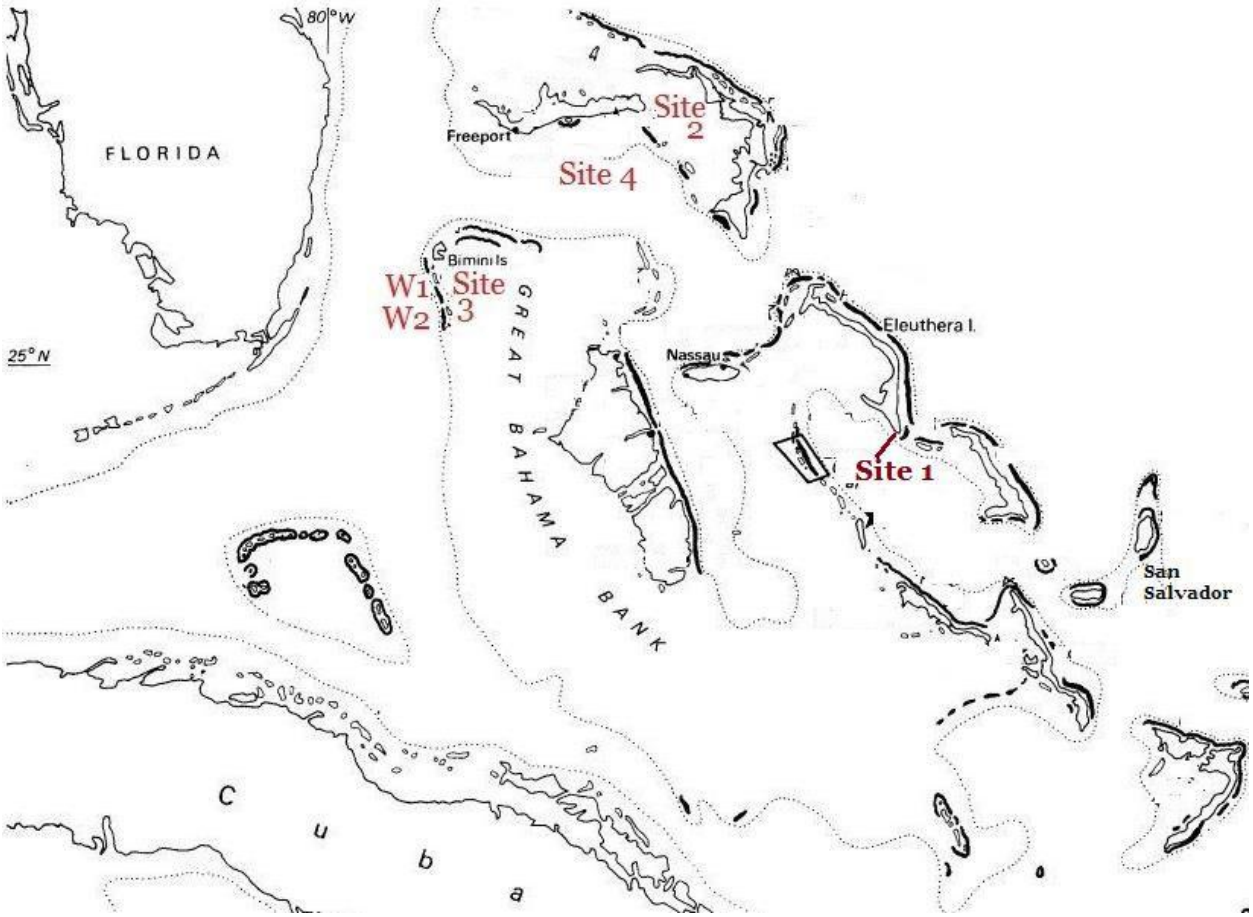


Figure 2.1: Map of the Bahamas showing reef locations and collection sites. Modified from the UNEP/IUCN.

Extraction of *P. elisabethae* holobiont genomic DNA

Genomic DNA (gDNA) was extracted from *P. elisabethae* using a phenol-chloroform protocol modified from Rowan and Powers (1991; 1992). Approximately 500 mg of *P. elisabethae* was frozen with liquid nitrogen and crushed using a sterile mortar and pestle. To avoid cross-contamination of DNA between samples, residual DNA was denatured from the mortar and pestles prior to use by soaking in 0.4 N NaOH for 30 minutes, rinsing well with MilliQ® water, and autoclaving. Five milliliters of freshly prepared DNA Lysis Buffer (DNAB, 25 mM EDTA, 25 mM Tris, 0.5 M NaCl, pH 8.0) was added to ground coral tissue and was transferred to a sterile centrifuge tube via a sterile pipette. Gentle mixing was performed throughout the extraction process to minimize shearing of high molecular weight DNA of both Gram-negative and Gram-positive bacteria (von Wintzingerode *et al*., 1997). Following brief centrifugation (100 x *g*, 2 min), the aqueous layer was transferred to a new 50 mL centrifuge tube containing 1 μL of RNase ONE (Promega, Madison, WI), 1 mg/mL lysozyme (Fischer Scientific, Ottawa, ON), 9 g/L polyvinylpyrrolidone, and sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. Bacterial gDNA was lysed from the cells by incubating the mixture at 37°C for 30 min. Proteinase K (Fischer Scientific, Ottawa, ON) was added to a final volume of 1 mg/mL and incubated at 55ºC for 2.5 hours. Following incubation, fine debris was removed by centrifugation (4,500 x *g* for 15 min). Supernatants were collected and sodium acetate was added to a final concentration of 0.3M, and DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Samples were gently inverted and centrifuged for 10 minutes at 9,800 x *g*. The aqueous phase was further extracted with an equal volume of chloroform:isoamyl alcohol (24:1) to remove trace phenol. Samples were centrifuged as above and the DNA was precipitated from the aqueous solution by the addition of 0.7 volumes of

isopropanol. Samples were centrifuged at 15,317 x *g* for 50 minutes at 4ºC, isopropanol was removed, and the DNA pellet washed with 5 mL of 70% ethanol (EtOH) to remove excess salts. Ethanol was decanted following storage on ice for five minutes, pellets were briefly dried in a laminar flow cabinet, and DNA was dissolved in 10 mM Tris-HCl (pH 8.0). To remove substances often co-extracted with genomic DNA from environmental samples which can interfere with subsequent PCR amplification, samples were cleaned using the PowerClean® DNA Clean-Up Kit (Mol Bio Laboratories, Carlsbad, CA) (samples 1A, 1B, 1D, 3E, 4A – 4C) or the Difficult Extraction Protocol (DEP, samples 1C, 1E, 2A – 2B, 3A – 3D) (Newman *et al*., 2010). DNA was extracted from agarose plugs in the DEP protocol using a QIAquick Gel Extraction Kit following the manufacturer's instructions. The integrity of all purified DNA was assessed by agarose gel electrophoresis (AGE).

16S rDNA amplification and preparation for bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP)

The diversity of bacteria associated with *P. elisabethae* was investigated by amplifying the universal 16S rRNA gene (Neefs *et al*., 1990). Bacterial 16S RNA gene sequences were amplified using the universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'), which target ~1,500 bp on the *E. coli* 16S rRNA gene (Edwards *et al*., 1989). PCR reactions (50 µL) contained 1X EconoTaq® Plus Green Master Mix (Lucigen, Middleton, WI, USA), 1 µM of each primer, 2.5% - 5% molecular biology grade DMSO (Sigma, Oakville, ON), and 10 – 30 ng template DNA. A hot start technique was used to minimize non-specific annealing of the primers to nontarget DNA (Muyzer *et al*., 1993). Thermal cycling parameters involved 1 cycle at 95ºC for 3 min, 34 cycles of 95ºC for 45 sec, 54ºC for 1 min, and 72ºC for 1.5 min and a final extension cycle at 72ºC for 10 min. Triplicate

PCR 16S rDNA amplicons from each coral holobiont gDNA sample were pooled to reduce potential amplification bias (Feinstein *et al*., 2009). PCR products were purified using a DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA) or following band excision and purification using a Zymoclean Gel DNA Recovery Kit (Zymo Research). Pooled PCR amplicons or selected gDNA samples were used as a template for bTEFAP.

16S rDNA amplicon pyrosequencing

Bacterial 16S rDNA amplicons from 13 healthy coral specimens representing 15 sample libraries in addition to two water libraries were sequenced by bTEFAP by Research and Testing Laboratories (RTL; Lubbock, TX). Titanium FLX amplicon pyrosequencing is similar to traditional bTEFAP technology, yet uses Titanium reagents, high-fidelity Taq polymerases, and a one-step PCR reaction (Wolcott *et al*., 2009). Binning of sequences from individual samples and cleaning of sequence libraries (removal of failed sequence reads, non-bacterial sequences, low quality sequences, chimeras, and sequence tags) was performed by RTL (Dowd *et al*., 2008). Failure of any sequence to meet quality control parameters resulted in sequence rejection. Approximately 500 bp of the V1 – V3 region on the 16S rRNA gene was amplified using 20 ng of template by the primer pair pA and 16S519r (5' – GAATTACCGCGGCGGCTG – 3') according to RTL procedures (Wolcott *et al*., 2009). This region was targeted for pyrosequencing as the information from partial sequencing of this region provides nearly equivalent taxonomic data to full-length sequences and also results in more accurate estimates of bacterial diversity, species richness, and bacterial community composition compared to other 16S rRNA gene variable regions (Huse *et al*., 2007; Kim *et al*., 2011). Due to the novelty of bTEFAP for inferring bacterial taxonomy in corals, method reproducibility and variability was assessed by A) comparison of the same PCR product (amplified in triplicate) amplified from the same coral

genomic DNA samples (sequence libraries 1A and 1B), sequenced once each in independent sequencing runs, and B) amplification of the V1 – V3 region from gDNA (sample 3A) and its respective nearly-full length 16S rDNA amplicon (sample 3B).

Taxonomic classification of bTEFAP sequences

Taxonomic classification of 16S rDNA sequence libraries was performed using the Ribosomal Database Project's pyrosequencing pipeline (RDP-II; Release 10, Cole *et al*., 2009), where PCR primer sequences (pA and 16S519r) and sequences containing ambiguous bases were removed. Sequences less than 250 bp were also discarded from the analysis as longer sequences result in more accurate taxonomic assignments and to reduce the possibility of obtaining artificially high richness estimations from short sequence reads (Engelbrektson *et al*., 2010; Wang *et al*., 2007). With 1,921,179 16S small-subunit rRNAs, the RDP has a large database suitable for taxonomically characterizing pyrosequencing data, and was selected owing to its ease of use and superiority in rapidly handling large pyrosequencing datasets.

To provide a compositional overview of the bacterial communities associated with *P. elisabethae* and water samples, the RDP clustering tool was used to cluster OTUs of 16S rRNA gene sequences at the "class" level (defined by 90% sequence similarity/10% sequence dissimilarity; $OTU_{0.10}$) (Wang *et al*., 2007). Similarly, bacterial 16S rDNA sequences were defined at the "species" level (97% sequence similarity; $OTU_{0.03}$) to determine the extent of influence individual phylotypes exert on shaping water and coral associated bacterial libraries. Confidence was inferred by the RDP using a 50% confidence threshold. This confidence threshold has previously been demonstrated to be suitable for accurate taxonomic assignment of short sequences and classifies a higher number of sequences than more stringent confidence thresholds (Claesson *et al*., 2009). Trimmed sequences were aligned by the RDP Sequence

Aligner, which aligns sequences based on an "infernal" (Interference of RNA Alignment) secondary-structure methodology (Nawrocki and Eddy, 2007). Although singletons may represent PCR artifacts, they were retained on the basis that they may represent rare taxa present in low levels in the sample (Meyer *et al*., 2009). Next, the RDP Classifier was used to identify sequences to the class level to provide the overall bacterial community composition (Cole *et al*., 2009). To compare the class-level community composition between samples, a percent composition chart based on RDP taxonomic classification was constructed.

Denaturing gradient gel electrophoresis (DGGE)

As inherent biases of pyrosequencing (Amend *et al*., 2010; Berry *et al*., 2011; Pinto and Raskin, 2012) may affect the ability of bTEFAP for assessing coral associated bacterial communities, denaturing gradient gel electrophoresis (DGGE) was selected as a complementary tool to analyze coral associated bacterial diversity. Following initial amplification of coral associated bacterial 16S rRNA genes described above, nested PCR was performed on selected corals using the modified primer 27f-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'), which contains a 40-nucleotide GC rich sequence (GC clamp) and primer P2 (Muyzer *et al*., 1993), which anneals to nucleotide positions 27 – 534 of the *E. coli* 16S rRNA gene and has an expected amplicon product of 507 bp. Amplification of the same nucleotide positions on the V1-V3 region in DGGE was chosen to maintain consistency between DGGE and bTEFAP to enable direct taxonomic comparison between sequences obtained from each method. Nested PCR reactions (50 µL) consisted of 1X EconoTaq® Plus Green Master Mix (Lucigen), 1 µM of each primer, 1µL of template DNA (16S rDNA amplification products as described previously), and 5% molecular biology grade DMSO. A touchdown thermal cycling protocol was used to prevent erroneous

sequencing priming during amplification (Don *et al*., 1991). Amplification consisted of the following parameters: 1 cycle at 95ºC for initial denaturing at 5 min, 10 cycles of 95ºC for 1 min, 66 - 56ºC (-1ºC/cycle) for 1 min, 72ºC for 2 min, 20 cycles of 95ºC for 1 min, 56ºC for 1.5 min, 72ºC for 2 min and 1 cycle at 72ºC for 7 min. Amplification of nested PCR products was confirmed by 1% AGE. For DGGE, 24 µL of each amplification product was loaded onto an 8% polyacrylamide gel containing a 70 – 40% urea-formamide denaturing gradient. Electrophoresis was performed using a D-Code system (Bio-Rad, Hercules, CA, USA) in 6.5L of 1 X TAE buffer. Gels were run at 60 V for 24 hours, stained with ethidium bromide, and dominant bands were excised, re-amplified by PCR using the above-stated primers and conditions, and re-run on the D-Code system to ensure purity. Prior to sequencing, DGGE bands were amplified using the primers pA and pD (Edwards *et al*., 1989). Amplicons were assessed for purity by 1% AGE, and the reamplified product representing DGGE bands were sequenced by Genome Québec (Montreal, QC, Canada) with primers pA and pD. Sequences were checked for chimeras using Bellerophon (Huber *et al*., 2004). Difficulties in PCR amplification prevented amplification for DGGE for all corals; hence, only successful nested amplicons were sequenced for samples 1B, 1C, 2A, and 3D.

Statistical analysis of coral-associated bacterial diversity

To describe and compare bacterial diversity between coral and water samples, the RDP pyrosequencing pipeline was used to calculate observed richness (S_{obs}), Chao1 richness estimates (S_{est}), Shannon's diversity index (*H'*) and evenness (*E*) (Cole *et al*., 2009). To calculate these statistics, 16S rDNA sequences were aligned using the RDP sequence Aligner (Cole *et al*., 2009). The resulting alignment files were used as the input files for the RDP Complete Linkage Clustering tool, which was used to identify OTUs at various sequence dissimilarities ($OTU_{0.10}$

and $OTU_{0.03}$). The resulting cluster file was used as the input file to calculate the aforementioned diversity statistics. To address sampling coverage, rarefaction curves were generated at the class and species level using the RDP Rarefaction calculator.

The RDP classifies pyrosequencing data to the genus level; however, in order to determine if particular bacterial species (defined at the conventional 97% sequence similarity, Cohan, 2002) were predominantly responsible for contributing to the variance in coral-associated bacterial communities, dominant $OTU_{0.03}$s were identified by BLAST sequence identities. Based on the high number of sequences retrieved, only dominant species-level phylotypes were analyzed. "Dominant phylotypes" herein are called "RKPE phylotypes" and defined as any $OTU_{0.03}$ contributing to ≥1% of an individual coral or water associated 16S rDNA sequence library. RKPE sequences were published in GenBank under the accession numbers shown in Table A1 (Appendix A). To provide the most accurate taxonomic estimation based on characterized bacterial cultures, taxonomic identification of RKPE phylotypes at $OTU_{0.03}$ was performed using GenBank's BLAST Reference RNA Sequence (RefSeq) database if sequences were >95% similar to published 16S rRNA gene sequences. However, RKPE phylotypes exhibiting ≤95% similarity to sequences in the RefSeq database in GenBank were identified using the BLASTn algorithm. DGGE band sequences were compared to respective coral pyrosequencing libraries using the Local BLASTn application in BioEdit (v7.0.9.0) (Hall, 1999).

Principal component analysis (PCA)

PCA was selected as a tool to model which $OTU_{0.03}$s were responsible for driving the separation of coral-associated bacterial libraries within and between reefs in the Bahamas. To prepare the data matrix a composite dataset containing sequences from all 16S rDNA sequence

libraries was prepared and the sequences aligned and clustered using the RDP as described above. The number of each $OTU_{0.03}$ present in each sample was tallied and the data was normalized by calculating the percent abundance of each $OTU_{0.03}$ per sample. Due to the high number of $OTU_{0.03}$s generated by pyrosequencing (9,036 $OTU_{0.03}$s from all samples), only dominant phylotypes ($OTU_{0.03}$s constituting ≥1% of an individual sequence library) were included in the PCA analysis. PCA was performed using the Unscrambler X (v10.1; Camo ASA, Oslo, Norway) using the single value decomposition algorithm and validated by cross validation.

Phylogenetic tree construction

To articulate the level of bacterial diversity associated with *P. elisabethae*, a phylogenetic tree of RKPE phylotype sequences was constructed. RKPE phylotypes (including water and coral $OTU_{0.03}$s) were aligned using ClustalW in MEGA 5.05 (Tamura *et al*., 2011). Aligned sequences were assessed to visualize phylogenetic relationships by a neighbour-joining phylogenetic tree (gaps treated by complete deletion) in MEGA 5.05, which best delineated S*ynechococcus* related phylotypes. Evolutionary distances were computed using the maximum composite likelihood method. *Aquifex pyrophilus* (M83548.2) was used to root the tree. Overall tree topologies were validated by reconstructing phylogenies using multiple evolutionary distance methods in MEGA 5.05, including maximum likelihood, minimum evolution, and unweighted pair group method with arithmetic mean (UPGMA, maximum composite likelihood).

RKPE23/Gven_K23 primer design and amplification from *P. elisabethae*

Due to the unusually high number and novelty of the RKPE23 gene sequence in sample 3E and sample 3A, a specific primer was designed to amplify this unique gene sequence on the

16S rRNA gene to screen other coral samples for the presence of these phylotypes. This sequence was not detected in any other sequence present in the RDP database except a clone from the octocoral *Gorgonia ventalina* (clone Gven_K23). RKPE23 and its closest GenBank match (Gven_K23, GU118339.1) were aligned with *E. coli* (J01695.2) and an unrelated *G. ventalina* clone (Gven_G10, GU118518.1) by the RDP sequence aligner. The RDP probe check was also used to test the specificity of the primer pair, yet only the Gven_K23 sequence could be found in the RDP database. Nested amplification on previously amplified 16S rRNA gene sequences (using the primers pA and pH) was performed on each *P. elisabethae* sample in order to determine the distribution of the Gven_K23/RKPE23 related phylotype in *P. elisabethae* corals. This novel sequence was used to design a 25 bp reverse primer. Nested amplification was performed on previously amplified 16S rRNA gene sequences (amplification described previously), and PCR was conducted as described for 16S rRNA gene sequence amplification (using the primer pair pA and Gven_K23-R1) with an annealing temperature of 51.1°C. A 203 bp amplicon was expected from successful amplifications. *E. coli* was amplified in conjunction with coral samples as a negative control.

PCR amplification of zooxanthellae DNA from selected *P. elisabethae* samples

Zooxanthellae DNA was amplified from coral homogenate gDNA using the primer pair D1/D2 (Zardoya *et al*., 1995), which provides the greatest resolution for zooxanthellae DNA (Sampayo *et al*, 2009). PCR reaction mixtures consisted of the following: 1X EconoTaq master mix, 1 µM of each primer (D1: 5'-CCCGCTGAATTTAAGCATATAAGTAAGCGG-3'; D2: 5'-GTTAGACTCCTTGGTCCGTGTTCAAGA-3'), 2 µL of DNA, 2.5% of molecular-biology grade DMSO, MilliQ® water to 25 µL. Thermal cycling parameters were 94°C for 3 min, 34 cycles of 94.0°C for 1 min, 65.0°C for 2 min, 72.0°C for 3 min; and a final extension of 72.0°C

for 5 min. Amplification was checked by 1% AGE, and sequencing was performed by Genome Québec using the primers D1/D2.

RESULTS AND DISCUSSION

Composition of *P. elisabethae* and water-associated bacterial communities

In this study, bTEFAP was employed as a tool to examine the bacterial community of 13 healthy specimens representing 15 sample libraries of the alcyonacean octocoral *P. elisabethae*, collected from four locations across the Bahamas. The results from select gDNA and PCR amplicons submitted for pyrosequencing are shown in Figure 2.2. In total, 120,550 sequences were generated from 15 coral-associated bacterial sequence libraries and 12,416 sequences were generated from two water samples. Sequence read lengths ranged from 373 – 454 bp, with an average sequence length of 432 bp (Table 2.1). Sequences could be assigned to 16 phyla and 29 classes, and a total of 9,035 $OTU_{0.03}$s were identified from the combined data set to describe species-level bacterial communities. The class-level bacterial community composition for coral and seawater samples is summarized in Figure 2.3. Although 29 bacterial classes were identified in octocoral-associated sequence libraries, the distribution of class-level taxa in individual libraries was highly variable; with 10-21 classes represented in individual sequences libraries. In contrast, 18 classes were identified from seawater with 15 and 14 classes being represented in W1 and W2 sequence libraries, respectively. As highlighted in Figure 2.3, all libraries were dominated by a few taxa, where the nine most abundant bacterial classes are shown while the less abundant taxa are grouped together in an arbitrary grouping labeled “remainder”. Here,

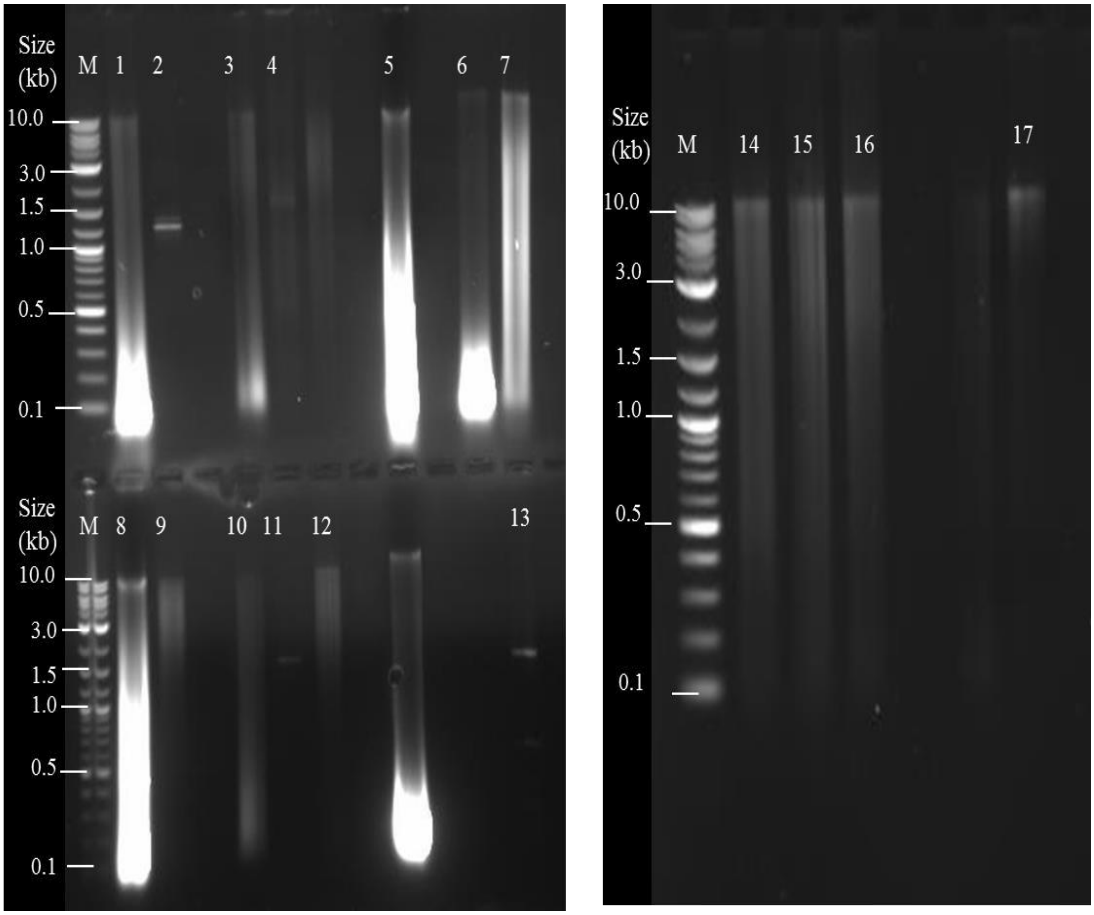


Figure 2.2: DNA extractions and selected PCR amplicons of *P. elisabethae* individuals. Band size molecular weights (in base pairs) are relative to the 2-log ladder (New England Biolabs). Coral sequence libraries are designated by a number and alphabetical letter as reflected in Table 2.1

Table 2.1: Sample collection information and sequencing and diversity statistics of 16S rDNA bTEFAP sequence libraries generated from *P. elisabethae* (PE) and seawater (W) DNA samples.

Sequence Library	Site[a]	Sample	Location	Latitude Longitude	Total Seq.	Avg. Length[b]	S_{obs}[c]	S_{est}[d]	H'[e]	E[f]
1A[g]	1	PE-A	Eleuthera	24° 48.55′ N 76° 20.58′ W	6979	436	659	977	4.2	0.65
1B[g]	1	PE-A	Eleuthera	24° 48.55′ N 76° 20.58′ W	8098	428	653	1072	4.2	0.65
1C	1	PE-C	Eleuthera	24° 48.55′ N 76° 20.58′ W	9511	440	508	669	3.9	0.62
1D	1	PE-D	Eleuthera	24° 48.55′ N 76° 20.58′ W	9330	373	1558	2510	6.0	0.82
1E	1	PE-E	Eleuthera	24° 48.55′ N 76° 20.58′ W	1207	440	15	17	0.9	0.34
2A	2	PE-A	Grand Bahama (Sweetings Cay)	26° 36.23′ N 77° 54.70′ W	10707	440	834	1315	4.0	0.60
2B	2	PE-B	Grand Bahama (Sweetings Cay)	26° 36.23′ N 77° 54.70′ W	11299	454	780	1169	3.9	0.58
3A[h]	3a	PE-A	Bimini (Victory Reef)	25° 29.13′ N 79° 16.41′ W	868	450	35	40	1.9	0.53
3B	3a	PE-A	Bimini (Victory Reef)	25° 29.13′ N 79° 16.41′ W	2387	451	135	244	2.3	0.46
3C	3a	PE-C	Bimini, (Victory Reef)	25° 29.13′ N 79° 16.41′ W	7510	452	635	1124	3.2	0.49
3D	3a	PE-D	Bimini (Victory Reef)	25° 29.13′ N 79° 17.95′ W	4195	435	871	1447	5.4	0.80
3E	3b*	PE-E	Bimini (Tuna Alley)	25° 31.38′ N 79° 16.41′ W	7793	382	935	1467	4.9	0.72
4A	4	PE-A	Grand Bahama (Peterson Reef)	26° 32.93′ N 78° 30.99′ W	8304	429	1344	1947	5.8	0.81
4B	4	PE-B	Grand Bahama (Peterson Reef)	26° 32.93′ N 78° 30.99′ W	10176	427	840	1096	4.8	0.71
4C	4	PE-C	Grand Bahama (Peterson Reef)	26° 32.93′ N 78° 30.99′ W	9707	447	478	604	3.4	0.55
W1	3a*	W	Bimini (Victory Reef)	25° 29.13′ N 79° 17.95′ W	4238	436	329	482	4.8	0.83
W2	3b*	W	Bimini (Tuna Alley)	25° 31.38′ N 79° 16.41′ W	8178	432	688	989	5.1	0.78

[a] Samples were collected in 2006 except for those marked with an asterisk which were collected in 2009

[b] Length in base pairs

[c] Richness at species-level distance (D = 0.03) based on observed unique OTUs.

[d] Chao1 non-parametric richness estimate at species-level distance (D = 0.03).

[e] Shannon diversity index at species-level distance (D = 0.03); a higher number indicates greater diversity.

[f] Evenness index; a higher number indicates greater evenness.

[g] Replicate bTEFAP sequence libraries prepared from the same genomic DNA sample.

[h] bTEFAP sequence library prepared using holobiont genomic DNA.

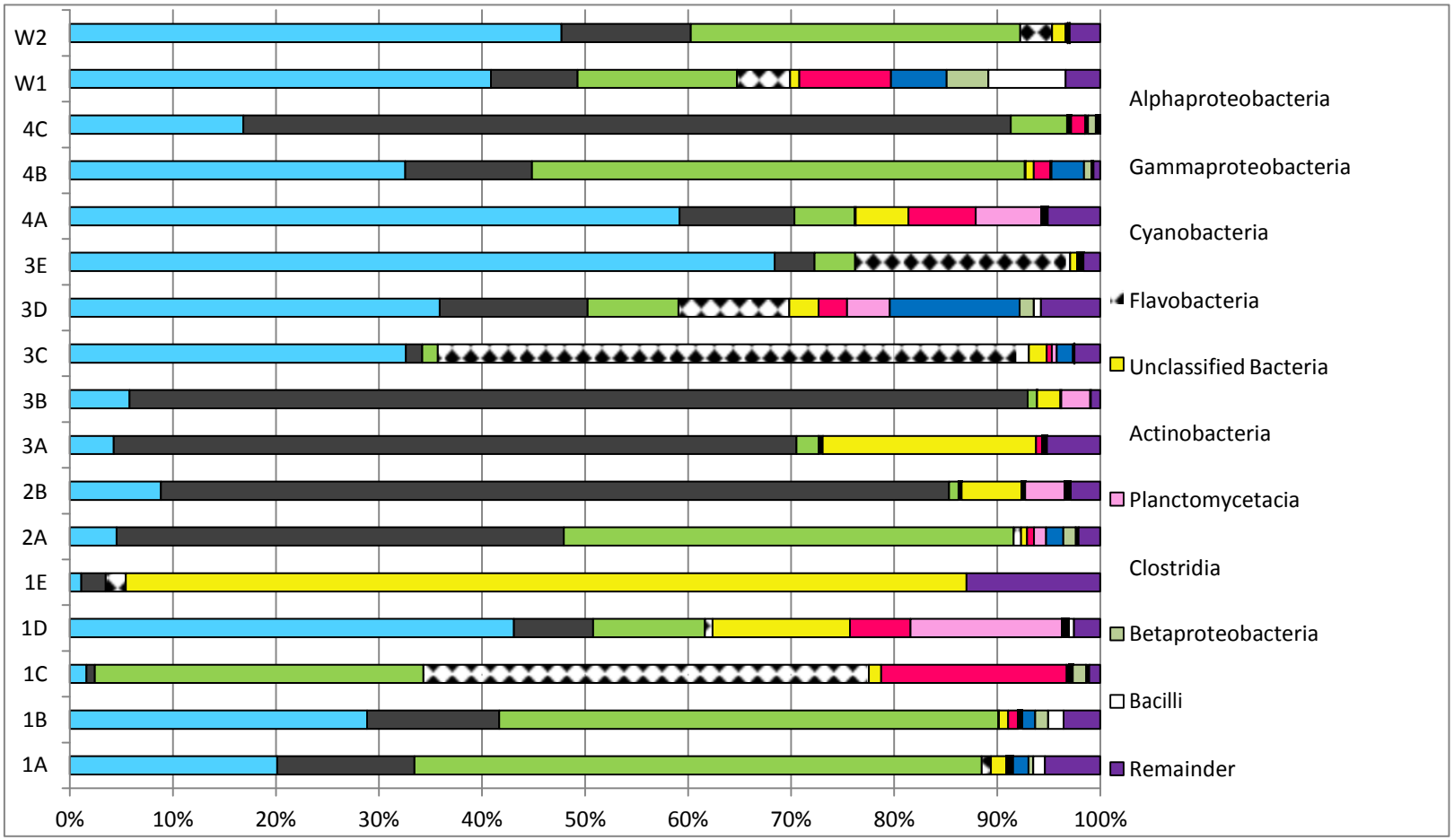


Figure 2.3: Class-level bacterial community composition of corals and seawater samples. Sequence libraries are represented on the Y-axis. "Unclassified Bacteria" refers to sequences which could not be classified by the RDP Classifier using a confidence threshold of 50%. "Remainder" is an artificial category encompassing relatively rare classes. This category accounts for 0.67-5.77% of each bacterial community.

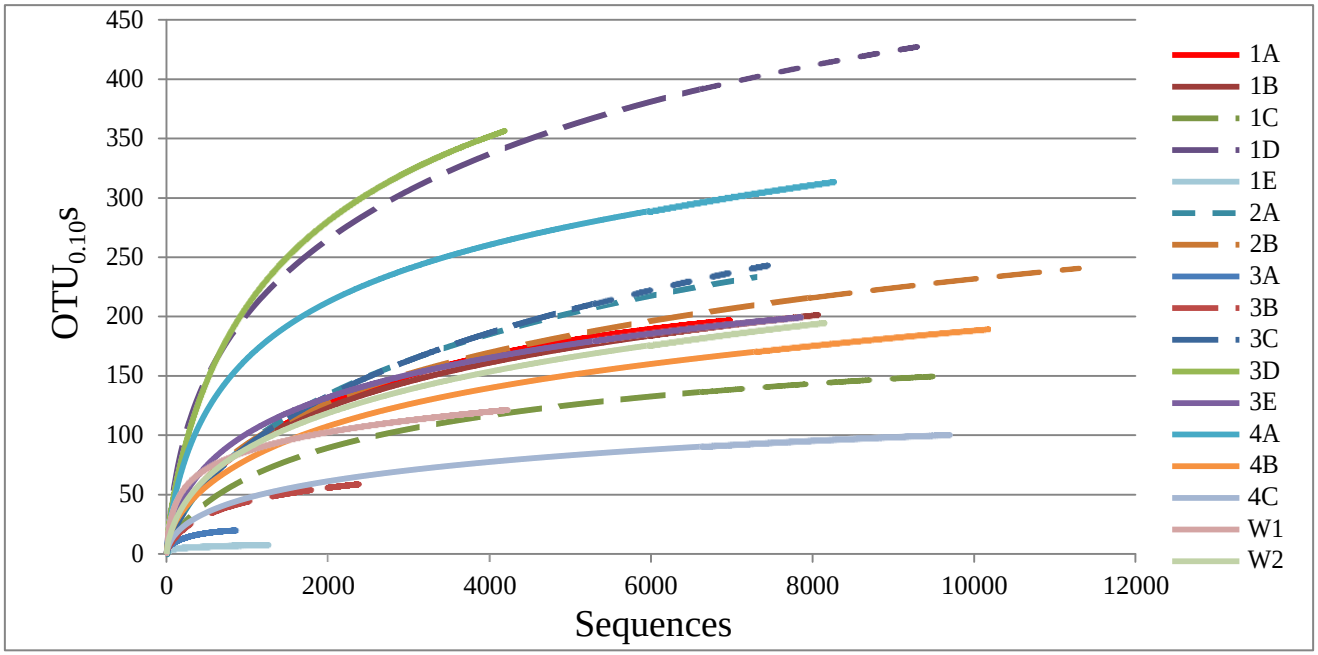
results depict that the relative abundance of bacterial classes varies substantially between corals collected at a single site (10-20 m separation) and between corals collected at different sites throughout the Bahamas (~60-300 km separation). Specifically, the bacterial community in *P. elisabethae* was dominated by Proteobacteria (Alpha and Gamma), Cyanobacteria, and in some sequence libraries, Flavobacteria (sample 1C, 3C, and 3E, 20.9 – 57.4%), a trend which is consistent with other culture-independent investigations of bacterial diversity from deep and shallow water octocorals (Brück *et al*., 2007, Penn *et al*., 2006; Sunagawa *et al*., 2010; Webster and Bourne, 2007). All corals contained at least a small percentage of sequences which could not be assigned to a bacterial phylum by the RDP Classifier using a 50% confidence threshold ("Unclassified Bacteria" Figure 2.3). These phylotypes were detected in the greatest abundance in sample 1E (81.5% of library) and 3A (23.0% of library), yet were also detected in samples 1D (<1%), 2B (3.3%), 3B (1.8%), 3C (<1%), and 3D (<1%). Bacterial communities of the water samples W1 and W2, which were separated by approximately 5 km, were both dominated by Alphaproteobacteria, Gammaproteobacteria and Cyanobacteria, yet W1 contained a greater abundance of Bacilli, Actinobacteria, Clostridia and Betaproteobacteria.

Bacterial diversity of *P. elisabethae* and surrounding water

To compare species-level ($OTU_{0.03}$) diversity between coral and water-associated bTEFAP libraries, non-parametric estimates of richness (Chao1) and diversity (Shannon diversity index-*H'*, evenness-*E*) were calculated (Table 2.1). Rarefaction curves were also prepared to estimate sampling coverage. Overall, *P. elisabethae*-associated bacterial communities were highly diverse, and with the exception of samples 1E, 3A, and 3B, most samples exhibited bacterial diversity comparable to the water samples. Genomic DNA libraries (sample 1E and 3A) in addition to the sample 3B community generated too few sequences to fully characterize bacterial

diversity, which may be a function of the low observed diversity (Table 2.1). In terms of observed (S_{obs}) and estimated richness (S_{est}), coral bacterial communities exhibited greater diversity (average: S_{obs} = 733, S_{est} =1120) than the water samples (average: S_{obs} = 509, S_{est} =535). Paradoxically, coral bacterial communities were comparable but somewhat less diverse than those of the water samples when considering average Shannon diversity index values (coral = 4.07, water = 4.95). Furthermore, 16S rRNA gene pyrosequences obtained from the water column were more even than corals (Table 2.1), a trend which has been confirmed in stony corals (Bourne and Munn 2005; Meron *et al*., 2011), sponges (Lee *et al*., 2009), and rock surfaces (Penn *et al*., 2006). The lower Shannon index values for coral bacterial communities can be explained by the dominance by a few taxa, a characteristic which is reflected in lower evenness (E) values for coral samples compared to water samples. As the Shannon index is sensitive to evenness, lower H' values can be expected from samples with less equal distribution of individual sequences between OTUs (Hill *et al*., 2003; Stirling and Wilsey, 2001). While most coral bacterial communities were highly diverse, the observed and estimated diversity of bacterial communities varied significantly between samples (Table 2.1). The greatest species-level ($OTU_{0.03}$) bacterial diversity in *P. elisabethae* in terms of both richness and evenness was observed in sequence libraries 1D, 3D, and 4A (5.4, 6.0, and 5.8 respectively), which is reflected in the high H' values for these samples. Conversely, samples 3A and 3B were the least diverse sequence libraries analyzed (1.9 and 2.3 respectively). In addition, most rarefaction curves failed to reach their asymptotic maxima at both the class and species level (Figure 2.4), indicating that deeper sampling is required to avoid underestimating species richness, particularly for samples 1D, 3D, and 4A.

A



B

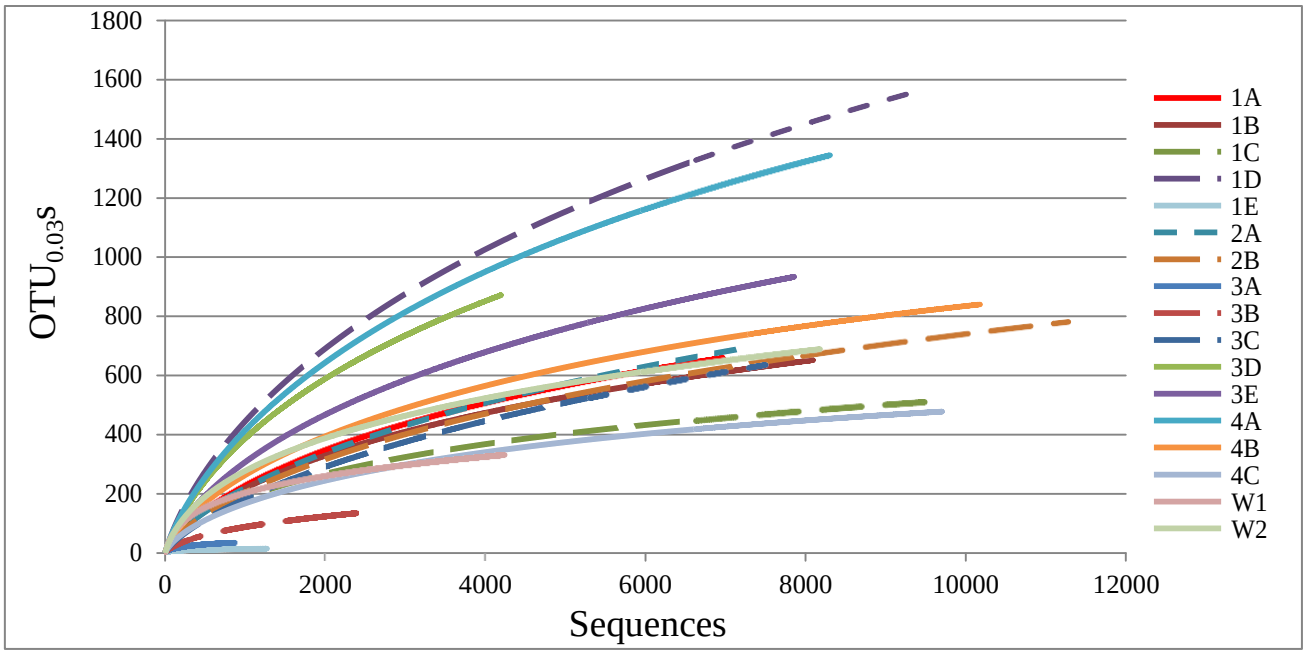
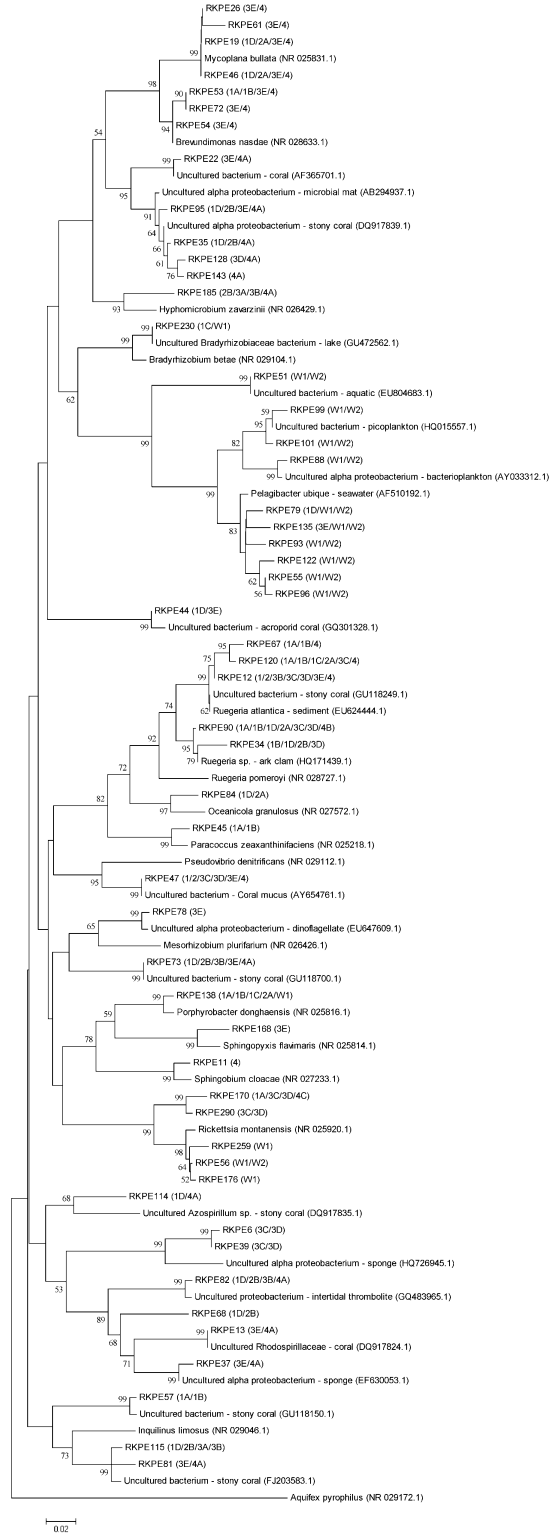


Figure 2.4: Rarefaction curves generated by the RDP-II from coral and water bTEFAP libraries at A) $OTU_{0.10}$s and B) $OTU_{0.03}$s. Refer to Table 2.1 for sequence library descriptions.

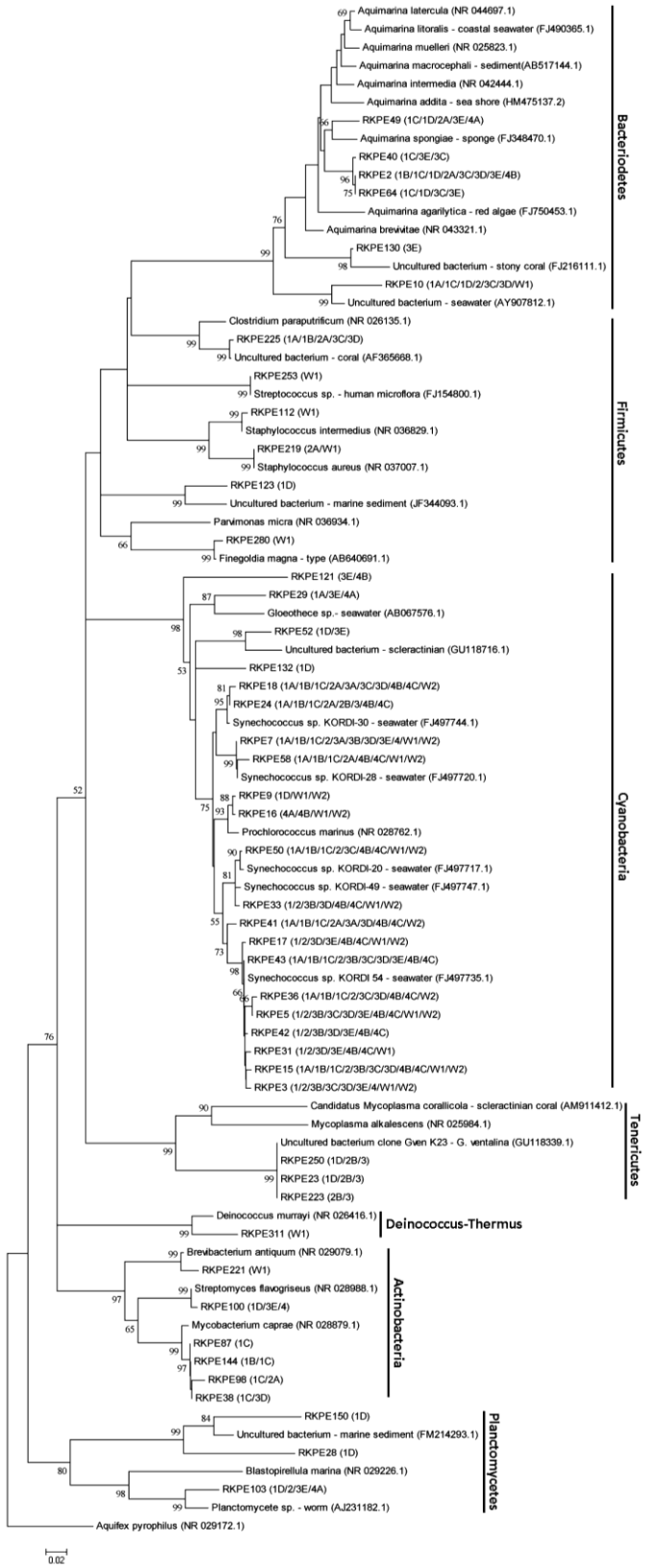
Dominant bacterial $OTU_{0.03}$s in *P. elisabethae*

To characterize the dominant species-level phylotypes shaping the overall bacterial community at the class level, 121 dominant $OTU_{0.03}$s ("dominant" defined as phylotypes present in ≥1% abundance in coral and water individual sequence libraries) from *P. elisabethae* were compared to published 16S rDNA sequences in the GenBank Database using the nucleotide BLASTn algorithm (Table A1). To determine the phylogenetic relationships between dominant phylotypes, a neighbour-joining phylogenetic tree was constructed (Figure 2.5). In Figure 2.5, the distribution of phylotypes throughout all sequence libraries is indicated in brackets after "RKPE". The presence of a sequence library in brackets indicates that the particular phylotype was found in that particular sequence library, yet it does not indicate that the phylotype was abundant. Overall, individual coral-associated sequence libraries differed in constitution and abundance between individual corals, while the most abundant $OTU_{0.03}$ varied between individuals. Although the overall bacterial community was not conserved, the occurrence and abundance of some phylotypes on corals sampled from different geographic locations suggests *P. elisabethae* has some level of host specificity. For example, no one specific $OTU_{0.03}$ was conserved across libraries in this study; however, RKPE3-related phylotypes (*Synechococcus* sp.) were consistently retrieved from all PCR-generated libraries and were conserved between corals and water, suggesting the presence in the coral is due to environmental uptake or contamination. Additionally, RKPE12 (*Ruegeria* sp.) was discovered in all PCR-generated coral libraries and was absent in the water column. Although the significance of this phylotype is unknown, cultured members of this genus have antibiotic activity (Nithya and Pandian, 2010); consequently, their ubiquitous presence across coral-associated bacterial libraries could suggest a role in defense against microbial pathogens. Additionally, the abundant "octocoral-specific"

A


RKPE26 (3E/4)
RKPE61 (3E/4)
RKPE19 (1D/2A/3E/4)
Mycoplana bullata (NR 025831.1)
RKPE46 (1D/2A/3E/4)
RKPE53 (1A/1B/3E/4)
RKPE72 (3E/4)
RKPE54 (3E/4)
Brevundimonas nasdae (NR 028633.1)
RKPE22 (3E/4A)
Uncultured bacterium - coral (AF365701.1)
Uncultured alpha proteobacterium - microbial mat (AB294937.1)
RKPE95 (1D/2B/3E/4A)
Uncultured alpha proteobacterium - stony coral (DQ917839.1)
RKPE35 (1D/2B/4A)
RKPE128 (3D/4A)
RKPE143 (4A)
RKPE185 (2B/3A/3B/4A)
Hyphomicrobium zavarzinii (NR 026429.1)
RKPE230 (1C/W1)
Uncultured Bradyrhizobiaceae bacterium - lake (GU472562.1)
Bradyrhizobium betae (NR 029104.1)
RKPE51 (W1/W2)
Uncultured bacterium - aquatic (EU804683.1)
RKPE99 (W1/W2)
Uncultured bacterium - picoplankton (HQ015557.1)
RKPE101 (W1/W2)
RKPE88 (W1/W2)
Uncultured alpha proteobacterium - bacterioplankton (AY033312.1)
Pelagibacter ubique - seawater (AF510192.1)
RKPE79 (1D/W1/W2)
RKPE135 (3E/W1/W2)
RKPE93 (W1/W2)
RKPE122 (W1/W2)
RKPE55 (W1/W2)
RKPE96 (W1/W2)
RKPE44 (1D/3E)
Uncultured bacterium - acroporid coral (GQ301328.1)
RKPE67 (1A/1B/4)
RKPE120 (1A/1B/1C/2A/3C/4)
RKPE12 (1/2/3B/3C/3D/3E/4)
Uncultured bacterium - stony coral (GU118249.1)
Ruegeria atlantica - sediment (EU624444.1)
RKPE90 (1A/1B/1D/2A/3C/3D/4B)
RKPE34 (1B/1D/2B/3D)
Ruegeria sp. - ark clam (HQ171439.1)
Ruegeria pomeroyi (NR 028727.1)
RKPE84 (1D/2A)
Oceanicola granulosus (NR 027572.1)
RKPE45 (1A/1B)
Paracoccus zeaxanthinifaciens (NR 025218.1)
Pseudovibrio denitrificans (NR 029112.1)
RKPE47 (1/2/3C/3D/3E/4)
Uncultured bacterium - Coral mucus (AY654761.1)
RKPE78 (3E)
Uncultured alpha proteobacterium - dinoflagellate (EU647609.1)
Mesorhizobium plurifarium (NR 026426.1)
RKPE73 (1D/2B/3B/3E/4A)
Uncultured bacterium - stony coral (GU118700.1)
RKPE138 (1A/1B/1C/2A/W1)
Porphyrobacter donghaensis (NR 025816.1)
RKPE168 (3E)
Sphingopyxis flavimaris (NR 025814.1)
RKPE11 (4)
Sphingobium cloacae (NR 027233.1)
RKPE170 (1A/3C/3D/4C)
RKPE290 (3C/3D)
Rickettsia montanensis (NR 025920.1)
RKPE259 (W1)
RKPE56 (W1/W2)
RKPE176 (W1)
RKPE114 (1D/4A)
Uncultured Azospirillum sp. - stony coral (DQ917835.1)
RKPE6 (3C/3D)
RKPE39 (3C/3D)
Uncultured alpha proteobacterium - sponge (HQ726945.1)
RKPE82 (1D/2B/3B/4A)
Uncultured proteobacterium - intertidal thrombolite (GQ483965.1)
RKPE68 (1D/2B)
RKPE13 (3E/4A)
Uncultured Rhodospirillaceae - coral (DQ917824.1)
RKPE37 (3E/4A)
Uncultured alpha proteobacterium - sponge (EF630053.1)
RKPE57 (1A/1B)
Uncultured bacterium - stony coral (GU118150.1)
Inquilinus limosus (NR 029046.1)
RKPE115 (1D/2B/3A/3B)
RKPE81 (3E/4A)
Uncultured bacterium - stony coral (FJ203583.1)
Aquifex pyrophilus (NR 029172.1)
0.02

B


Aquimarina latercula (NR 044697.1)
Aquimarina litoralis - coastal seawater (FJ490365.1)
Aquimarina muelleri (NR 025823.1)
Aquimarina macrocephali - sediment(AB517144.1)
Aquimarina intermedia (NR 042444.1)
Aquimarina addita - sea shore (HM475137.2)
RKPE49 (1C/1D/2A/3E/4A)
Aquimarina spongiae - sponge (FJ348470.1)
RKPE40 (1C/3E/3C)
RKPE2 (1B/1C/1D/2A/3C/3D/3E/4B)
RKPE64 (1C/1D/3C/3E)
Aquimarina agarilytica - red algae (FJ750453.1)
Aquimarina brevivitae (NR 043321.1)
RKPE130 (3E)
Uncultured bacterium - stony coral (FJ216111.1)
RKPE10 (1A/1C/1D/2/3C/3D/W1)
Uncultured bacterium - seawater (AY907812.1)
Bacteriodetes
Clostridium paraputrificum (NR 026135.1)
RKPE225 (1A/1B/2A/3C/3D)
Uncultured bacterium - coral (AF365668.1)
RKPE253 (W1)
Streptococcus sp. - human microflora (FJ154800.1)
RKPE112 (W1)
Staphylococcus intermedius (NR 036829.1)
RKPE219 (2A/W1)
Staphylococcus aureus (NR 037007.1)
RKPE123 (1D)
Uncultured bacterium - marine sediment (JF344093.1)
Parvimonas micra (NR 036934.1)
RKPE280 (W1)
Finegoldia magna - type (AB640691.1)
Firmicutes
RKPE121 (3E/4B)
RKPE29 (1A/3E/4A)
Gloeothece sp.- seawater (AB067576.1)
RKPE52 (1D/3E)
Uncultured bacterium - scleractinian (GU118716.1)
RKPE132 (1D)
RKPE18 (1A/1B/1C/2A/3A/3C/3D/4B/4C/W2)
RKPE24 (1A/1B/1C/2A/2B/3/4B/4C)
Synechococcus sp. KORDI-30 - seawater (FJ497744.1)
RKPE7 (1A/1B/1C/2/3A/3B/3D/3E/4/W1/W2)
RKPE58 (1A/1B/1C/2A/4B/4C/W1/W2)
Synechococcus sp. KORDI-28 - seawater (FJ497720.1)
RKPE9 (1D/W1/W2)
RKPE16 (4A/4B/W1/W2)
Prochlorococcus marinus (NR 028762.1)
RKPE50 (1A/1B/1C/2/3C/4B/4C/W1/W2)
Synechococcus sp. KORDI-20 - seawater (FJ497717.1)
Synechococcus sp. KORDI-49 - seawater (FJ497747.1)
RKPE33 (1/2/3B/3D/4B/4C/W1/W2)
RKPE41 (1A/1B/1C/2A/3A/3D/4B/4C/W2)
RKPE17 (1/2/3D/3E/4B/4C/W1/W2)
RKPE43 (1A/1B/1C/2/3B/3C/3D/3E/4B/4C)
Synechococcus sp. KORDI 54 - seawater (FJ497735.1)
RKPE36 (1A/1B/1C/2/3C/3D/4B/4C/W2)
RKPE5 (1/2/3B/3C/3D/3E/4B/4C/W1/W2)
RKPE42 (1/2/3B/3D/3E/4B/4C)
RKPE31 (1/2/3D/3E/4B/4C/W1)
RKPE15 (1A/1B/1C/2/3B/3C/3D/4B/4C/W1/W2)
RKPE3 (1/2/3B/3C/3D/3E/4/W1/W2)
Cyanobacteria
Candidatus Mycoplasma corallicola - scleractinian coral (AM911412.1)
Mycoplasma alkalescens (NR 025984.1)
Uncultured bacterium clone Gven K23 - G. ventalina (GU118339.1)
RKPE250 (1D/2B/3)
RKPE23 (1D/2B/3)
RKPE223 (2B/3)
Tenericutes
Deinococcus murrayi (NR 026416.1)
RKPE311 (W1)
Deinococcus-Thermus
Brevibacterium antiquum (NR 029079.1)
RKPE221 (W1)
Streptomyces flavogriseus (NR 028988.1)
RKPE100 (1D/3E/4)
Mycobacterium caprae (NR 028879.1)
RKPE87 (1C)
RKPE144 (1B/1C)
RKPE98 (1C/2A)
RKPE38 (1C/3D)
Actinobacteria
RKPE150 (1D)
Uncultured bacterium - marine sediment (FM214293.1)
RKPE28 (1D)
Blastopirellula marina (NR 029226.1)
RKPE103 (1D/2/3E/4A)
Planctomycete sp. - worm (AJ231182.1)
Planctomycetes
Aquifex pyrophilus (NR 029172.1)
0.02

C

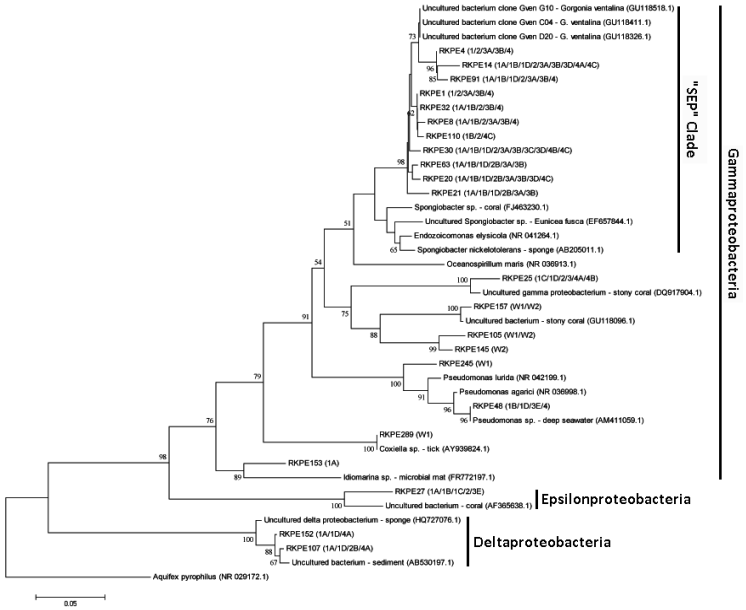


Figure 2.5: Neighbour-joining phylogenetic tree of RKPE sequences. Evolutionary distances were calculated using the maximum composite likelihood method. (A) Alphaproteobacteria; (B) Bacteriodetes, Firmicutes, Cyanobacteria, Tenericutes, Deinococcus-Thermus, Actinobacteria and Plancotmycetes; (C) other Proteobacteria of dominant phylotypes from *P. elisabethae* and seawater samples. Bootstrapping (n = 1,000) was used to assess overall tree accuracy, and aligned nucleotide sequence gaps were treated by complete deletion. Only bootstrap probabilities ≥50% are shown. The scale bars represent five nucleotide (nt) substitutions per 100 nt positions for (A) and (B) and two nt substitutions per 100 nt positions for (C). All trees were rooted with *Aquifex pyrophilus* (NR_029172.1). The sequence libraries in which phylotypes were present (but not necessarily abundant) are indicated in brackets following the RKPE number. Numbers in brackets which are not followed by a letter indicate that all sequence libraries starting with that number contain representatives of that particular phylotype. GenBank accession numbers for reference strains are shown in parentheses. Classes are indicated on side bars; "SEP" refers to *Spongiobacter-Endozoicomonas* phylotypes in the class Gammaproteobacteria.

phylotypes RKPE1 (Gammaproteobacteria) and RKPE23 (Unclassified Bacteria) were also absent in the water column; the significance of these phylotypes will be discussed in detail subsequently.

Different bacterial classes dominated individual coral-associated bacterial libraries at the species level. For example, Flavobacteria (*Aquimarina* sp.) dominated sequence libraries 1C and 3C, an uncultured Planctomycetacia "dominated" sample 1D (5.6% abundance), and an uncultured Alphaproteobacteria "dominated" sample 4A (4.9% abundance; Table 2.2). In coral samples 2A, 2B, 3A, 3B, and 4C, Gammaproteobacteria were dominated by RKPE1-related phylotypes (Table 2.2; Figure 2.5). These phylotypes were most similar to *Endozoicomonas* sp. (unpublished pseudonym *Spongiobacter*), which also included a sequence obtained with high similarity to an uncultured bacterium from the octocoral *Gorgonia ventalina* (clone Gven_G10, GU118518.1, 98% BLAST sequence identity) and a clone from the Mediterranean octocoral *Eunicella cavolini* (clone B10, JQ691564.1, Table A1, Appendix). Sequences were more similar to *G. ventalina* clone Gven_G10 than *Spongiobacter* (≤95% sequence identity), and hence all RKPE1-related phylotypes in Table 2.2 are compared to the *G. ventalina* phylotype. RKPE23-related phylotypes dominated sequence library 1E (83.8%) and to some extent, sample 3A (32.1% of phylotypes contributing to ≥1% of library) while samples 1D, 2A, 2B, and all Victory Reef libraries (sample 3A – 3D) contained these phylotypes yet in reduced abundance. These phylotypes were not classified into a bacterial phylum based on the RDP Classifier and shared 80% sequence identity with *Mycoplasma alkalescens* in the phylum Tenericutes (NR025984.1). However, these phylotypes most closely resembled an uncultured *G. ventalina* clone Gven_K23 (GU118339.1; 100% identity), the significance of which will be discussed in detail subsequently.

Table 2.2: Dominant $OTU_{0.03}$s from coral and water-associated bacterial sequence libraries.

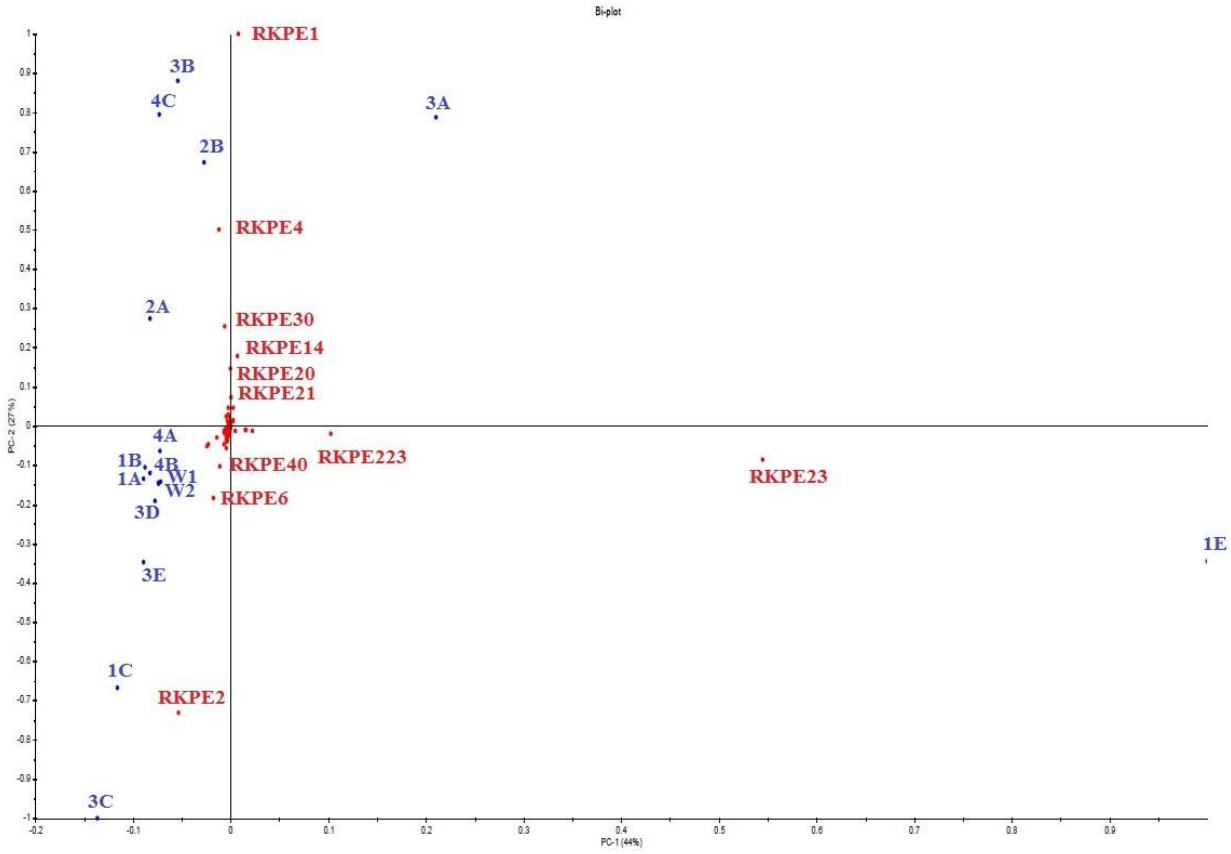
Site	% of library	$OTU_{0.03}$	Closest GenBank Match (Accession number; percent similarity)	Class
1A	13.9	RKPE3	*Synechococcus* sp. KORDI-54 (FJ497735.1; 99%)	Cyanobacteria
1B	13.6	RKPE3	*Synechococcus* sp. KORDI-54 (FJ497735.1; 99%)	Cyanobacteria
1C	24.5	RKPE2	*Aquimarina* sp. (HM998909.1; 94%)	Flavobacteria
1D	5.6	RKPE28	Uncultured Planctomycetales (FJ516934.1; 92%)	Planctomycetacia
1E	83.8	RKPE23, RKPE223	Uncultured *Gorgonia ventalina* clone Gven_K23 (GU118339.1; 98%)	Unclassified Bacteria
2A	17.3	RKPE1	Uncultured *Gorgonia ventalina* clone Gven10 (GU118518.1; 98%)	Gammaproteobacteria
2B	25.6	RKPE1	Uncultured *Gorgonia ventalina* clone Gven10 (GU118518.1; 98%)	Gammaproteobacteria
3A	32.1	RKPE1	Uncultured *Gorgonia ventalina* clone Gven10 (GU118518.1; 98%)	Gammaproteobacteria
3B	31	RKPE1	Uncultured *Gorgonia ventalina* clone Gven10 (GU118518.1; 98%)	Gammaproteobacteria
3C	37.9	RKPE2	*Aquimarina* sp. (HM998909.1; 94%)	Flavobacteria
3D	9.5	RKPE25	Uncultured Gammaproteobacterium (DQ917904.1; 93%)	Gammaproteobacteria
3E	10.8	RKPE13	Uncultured bleached *Muricea elongata* Rhodospirillaceae bacterium (DQ917824; 99%)	Alphaproteobacteria
4A	4.9	RKPE35	Uncultured Alphaproteobacterium (DQ917839.1; 97%)	Alphaproteobacteria
4B	7.8	RKPE3	*Synechococcus* sp. KORDI-54 (FJ497735.1; 99%)	Cyanobacteria
4C	31.3	RKPE1	Uncultured *Gorgonia ventalina* clone Gven10 (GU118518.1; 98%)	Gammaproteobacteria
W1	6.5	RKPE56	*Rickettsia monacensis* (DQ100164.1; 99%)	Alphaproteobacteria
W2	10.6	RKPE9	*Prochlorococcus marinus* (CP000576.1; 96%)	Cyanobacteria

Characterizing dominant phylotypes driving the separation of coral-associated bacterial communities by PCA

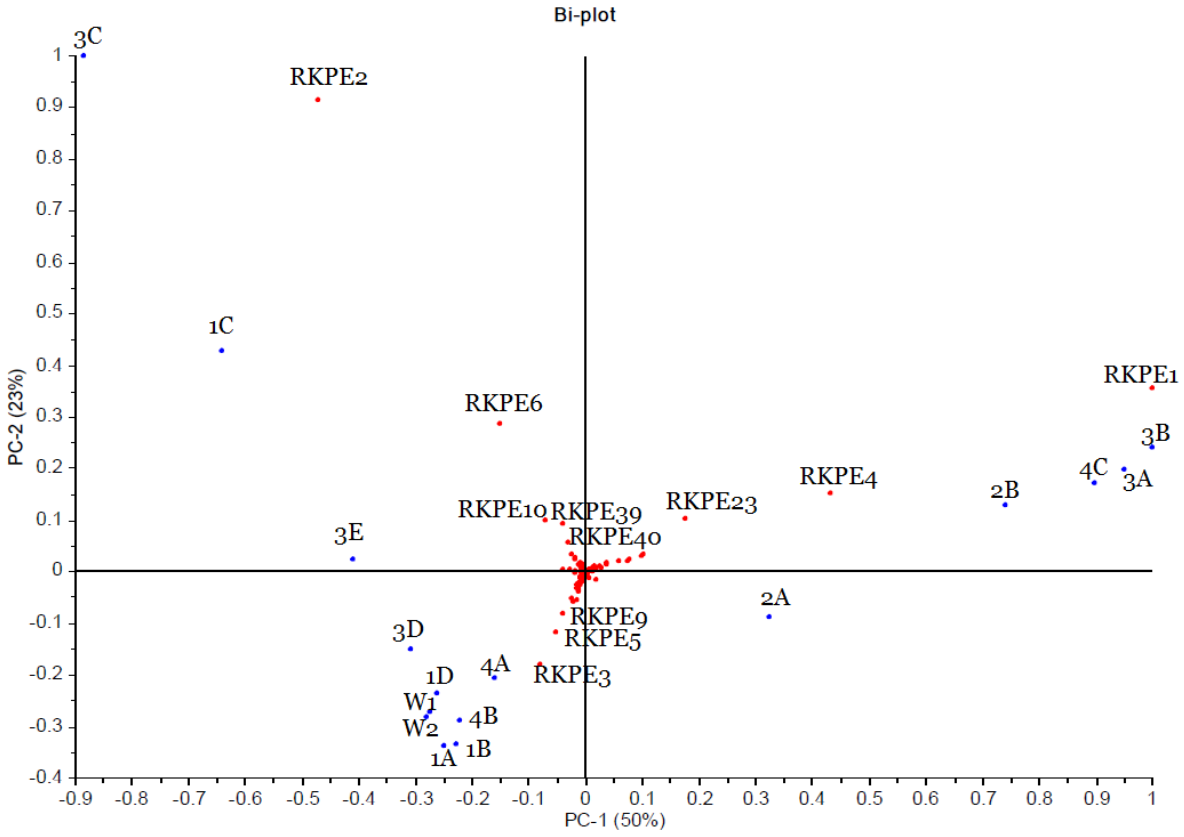
Principal component analysis (PCA) was performed to visualize relationships between coral and water-associated bacterial communities and to determine bacterial phylotypes ($OTU_{0.03}$s) responsible for the variation observed between coral and water-associated bacterial communities. The basic premise of PCA is that samples containing similar dominant variables (in this case, members of a bacterial community as measured by the abundance of clusters at $OTU_{0.03}$) will migrate along one principal component (PC) while those having different dominant variables will separate away from each other. Each principal component represents the line of best fit through n-dimensional space (n = number of variables) which explains the most variance within the data. Each subsequent principal component is orthogonal to the previous component and describes sequentially less variance.

The abundance of the RKPE23/223 phylotypes heavily skewed the data for the main PCA biplot when sample 1E was included in the analysis (Figure 2.6A). As a result, sample 1E was excluded from the main PCA biplot as an outlier in order to visualize the other factors affecting the PCA model (Figure 2.6B, C). An explained variance plot was generated for sequence libraries excluding sample 1E, and demonstrated that the model optimized after 4 PCs and explained 86% of the model variance (PC1 explains 50%, PC2 explains 23%, PC3 explains 9%, and PC4 explains 4%). Further principal components (PCs 5-7) continued to explain decreasingly less of the model variance (3%, 3%, and 2% respectively) and did not correspond to major differences within the data structure.

A


Bi-plot
RKPE1
3B
4C
3A
2B
RKPE4
2A
RKPE30
RKPE14
RKPE20
RKPE21
4A
1B
4B
1A
W1
W2
RKPE40
RKPE223
RKPE6
3D
RKPE23
1E
3E
1C
RKPE2
3C
PC-2 (27%)
PC-1 (44%)

B


Bi-plot
PC-2 (23%)
PC-1 (50%)
3C
RKPE2
1C
RKPE6
RKPE1
3B
4C
3A
RKPE4
2B
RKPE10
RKPE39
RKPE23
RKPE40
3E
2A
RKPE9
RKPE5
3D
4A
1D
RKPE3
W1
W2
4B
1A
1B

C

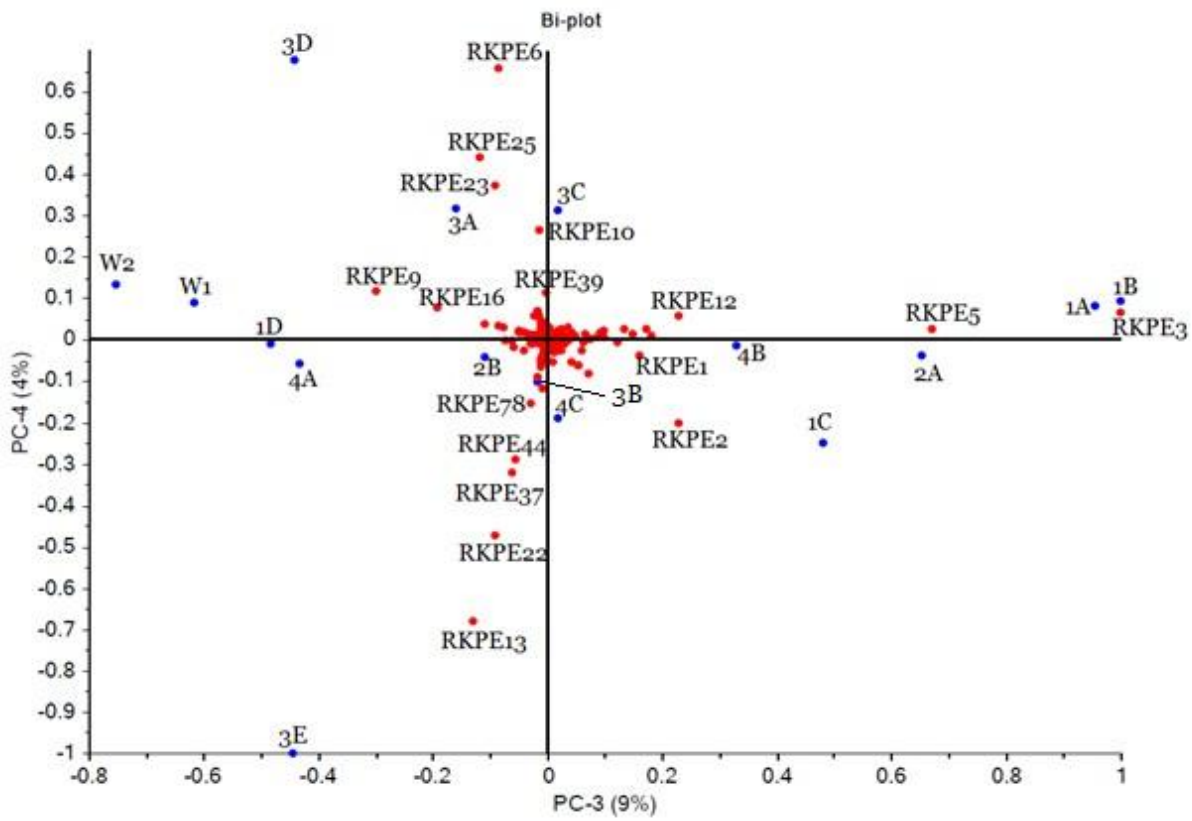


Figure 2.6: Principal Component Analysis biplots describing dominant bacterial $OTU_{0.03}$s responsible for the observed variance between the bacterial communities of corals and water. (A) PCA biplot of PCs 1 – 2 including sample 1E. Excluding sample 1E, PC1 and 2 (B) describe 50% and 23% respectively of the observed variance while PC3 and 4 (C) describes an additional 9 and 4% of the variance respectively, explaining 86% of the total variance. Blue dots represent coral and water samples while blue dots represent dominant 16S rRNA gene sequence phylotypes at $OTU_{0.03}$.

The majority of the model variance was explained within the first two principal components (PC1 50% and PC2 23%; Figure 2.6B). PC1 and PC2 distinguish bacterial communities dominated by Gammaproteobacteria (specifically RKPE1 and RKPE4, visualized by positive separation along PC1) and Flavobacteria (RKPE2, visualized by negative separation along PC1 and positive separation along PC2). Strong positive separation along PC1 directly correlated with the abundance of the Gammaproteobacteria-related phylotypes RKPE1 and RKPE4 within the bacterial communities of libraries from sample 2A, 2B, 3A/B, and 4C. From the phylogenetic tree (Figure 2.5), these phylotypes correlated to a clade clustering together with several uncultured Gammaproteobacteria phylotypes previously reported from *Gorgonia ventalina* clones Gven_C04 (GU118411.1), Gven_D20 (GU118326.1) and Gven_G10 (GU118518.1; Sunagawa *et al*., 2010) and to recently published sequences in GenBank from the Mediterranean octocoral *Eunicella cavolini* (ex. clone B10, JQ691564.1; 98%). Comparison of RKPE1-related phylotypes to characterized strains in GenBank returned matches most closely related to *Spongiobacter nickelotolerans* (93% similarity) and *Endozoicomonas elysicola* (91% similarity). The overall similarity of the replicate sequence libraries (1A and 1B) was confirmed in the PCA model as both samples demonstrated comparable trends (Figure 2.6B, C). Water libraries W1 and W2 also demonstrated similar trends to each other, as did the Bimini sequence libraries generated from gDNA (3A) and the PCR amplicon (3B) bTEFAP templates, with the exception of PC4 due to the high abundance of phylotype RKPE23 (unclassified bacterium) in 3A compared to 3B. RKPE1-related phylotypes were dominant in some corals and were generally conserved between *P. elisabethae* samples in the Bahamas (Table 2.3).

On the PCA biplot, the dominance of Flavobacteria within the coral-associated bacterial communities is modeled by the negative separation along PC1 and positive separation along PC2

Table 2.3: Population abundance of "Gven_G10" clade phylotypes as an individual percent abundance, total percent abundance and relative percent abundance to total Gammaproteobacteria community of individual coral and water samples. Bold type indicates phylotypes that had the most influence on the PCA model.

PHYLOTYPE	1A	1B	1C	1D	2A	2B	3A	3B	3C	3D	3E	4A	4B	4C	W1	W2
RKPE1	**1.4**	**1.8**	**0**	**0**	**17.3**	**25.6**	**32.1**	**31.0**	**0**	**0**	**0**	**2.6**	**1.8**	**31.3**	**0**	**0**
RKPE4	**0.7**	**1.6**	**0**	**0**	**5.9**	**12.6**	**11.4**	**15.0**	**0**	**0**	**0**	**1.3**	**0.7**	**14.3**	**0**	**0**
RKPE8	0.1	0.2	0	0	3.5	2.7	1.3	3.6	0	0	0	0.3	0.2	4.0	0	0
RKPE14	1.0	1.0	0	0.2	0.2	4.0	3.1	5.9	0	0.1	0	0	0	0.3	0	0
RKPE20	0.8	1.1	0	0.1	0	3.1	1.8	5.3	0	0	0	0	0	0	0	0
RKPE21	1.1	1.2	0	0.1	0	2.6	2.6	4.6	0	0	0	0	0	0	0	0
RKPE30	0.6	0.6	0	0.1	0.2	2.2	2.1	3.1	0	0.1	0	0	0	0.3	0	0
RKPE32	0.1	0.1	0	0	0.8	1.2	0	0.6	0	0	0	0.2	0.1	1.6	0	0
RKPE63	0.3	0.3	0	0.1	0	1.2	2.1	1.6	0	0	0	0	0	0	0	0
RKPE91	0.1	0	0	0	0.2	0.4	0.9	1.0	0	0	0	0	0	0.5	0	0
RKPE110	0	0	0	0	1.0	0.1	0	0	0	0	0	0	0	0.1	0	0
Total % Abundance	6.1	7.8	0	0.5	29.0	55.6	57.4	71.7	0	0.2	0	4.4	2.8	52.4	0	0
Relative % Gammaproteobacteria Abundance	45.9	60.9	0	6.5	66.8	72.7	86.7	82.2	0	1.4	0	39.3	22.8	70.3	0	0

for coral samples 3C, 1C, and 3E (Figure 2.6B). Negative separation along PC1 of samples sample 3C, 1C and to lesser extent, sample 3E are due to the absence of SEPs while positive separation along PC2 is correlated with the presence of the RKPE2 phylotype within the bacterial communities of these corals. The RKPE2 phylotype clustered together in an *Aquimarina* spp. clade within the Flavobacteria along with RKPE49, RKPE40, and RKPE64. These phylotypes made up the majority of the abundance of Flavobacteria-related sequences observed in samples 1C, 3C, and 3E (71.8%, 69.9% and 50.7% respectively; Table 2.4).

Separation of samples along PC3 (Figure 2.6C) describes the variation in composition of dominant Cyanobacteria present in the dominant bacterial communities. Positive separation of sequence libraries 1A/B, 1C, 2A, and 4B along PC3 correlated with the relative abundance of RKPE3 and RKPE5 phylotypes within those communities. Phylotype RKPE3 and RKPE5 formed a clade with phylotypes RKPE15, 17, 31, 36, and 41-43 and *Synechococcus* sp. KORDI 54 (FJ497735.1, 99% sequence identity). These closely-related phylotypes represented 33.7%/30.7%, 15.6%, 21.8% and 19.0% of the total bacterial population and 61.2%/63.4%, 48.9%, 49.9% and 39.7% of the dominant Cyanobacteria $OTU_{0.03}$s in samples 1A/1B, 1C, 2A and 4B, respectively (Table 2.5). *Synechococcus* phylotypes were observed in all but one coral sequence library (4A) and were present in seawater libraries (0.2 – 1.2% overall abundance; Table 2.5). Negative separation along PC3 correlated with the low abundance of RKPE3/5 clade phylotypes in corals and a high abundance of phylotypes RKPE9 and RKPE16 in water samples (Figure 2.6C). RKPE9 and RKPE16 formed a unique clade with a *Prochlorococcus marinus* subsp. *pastoris* strain (NR 028762.1, 98% sequence similarity), which were rare phylotypes obtained from corals (detected in <0.04% abundance in samples 1D, 4A, and 4B) yet were

Table 2.4: Population abundance of "*Aquimarina* spp." clade phylotypes as an individual percent abundance, total percent abundance and relative percent abundance to total Flavobacteria community of individual coral and water samples. Bold type indicates phylotypes that had the most influence on the PCA model.

PHYLOTYPE	1A	1B	1C	1D	2A	2B	3A	3B	3C	3D	3E	4A	4B	4C	W1	W2
RKPE2	**0**	**0**	**24.5**	**0.2**	**0**	**0**	**0**	**0**	**37.9**	**0**	**9.0**	**0**	**0**	**0**	**0**	**0**
RKPE40	0	0	1.8	0	0	0	0	0	2.2	0	0.6	0	0	0	0	0
RKPE49	0	0	3.1	0	0.2	0	0	0	0	0	0.1	0	0	0	0	0
RKPE64	0	0	0.8	0	0	0	0	0	1.1	0	0.9	0	0	0	0	0
Total % Abundance	0	0	30.2	0.2	0.2	0	0	0	41.2	0	10.6	0	0	0	0	0
Relative % Flavobacteria Abundance	0	0	69.9	26.3	27.5	0	0	0	71.8	0	50.7	0	0	0	0	0

Table 2.5: Population abundance of "*Synechococcus* sp. KORDI-54" clade phylotypes as an individual percent abundance, total percent abundance and relative percent abundance to total Cyanobacteria community of individual coral and water samples. Bold type indicates phylotypes that had the most influence on the PCA model.

PHYLOTYPE	1A	1B	1C	1D	2A	2B	3A	3B	3C	3D	3E	4A	4B	4C	W1	W2
RKPE3	**13.9**	**13.6**	**5.7**	**0.1**	**8.9**	**0.1**	**0**	**0.4**	**0.4**	**1.1**	**0.3**	**0**	**7.8**	**0.7**	**0.6**	**0.1**
RKPE5	**8.5**	**9.3**	**4.1**	**0**	**6.9**	**0.2**	**0**	**0**	**0.2**	**0.6**	**0.2**	**0**	**4.8**	**0.5**	**0.3**	**0.1**
RKPE15	2.6	2.4	1.2	0	1.6	0.1	0	0	0.1	0.1	0	0	0.9	0.1	0	0
RKPE17	3.6	1.1	0.4	0	0.5	0.1	0	0	0	0	0.1	0	2.0	0.3	0.3	0
RKPE31	1.6	1.2	0.5	0	0.6	0	0	0	0	0.1	0	0	1.2	0.1	0	0
RKPE36	1.0	1.2	0.6	0	0.8	0	0	0	0.1	0.1	0	0	0.6	0.1	0	0
RKPE41	0.3	0.3	2.0	0	1.0	0	0.1	0	0	0.1	0	0	0.2	0.1	0	0
RKPE42	1.0	0.9	0.5	0	0.8	0	0	0	0	0.1	0	0	0.7	0.2	0	0
RKPE43	1.2	0.7	0.6	0	0.7	0	0	0	0	0.1	0	0	0.8	0.1	0	0
Total % Abundance	33.7	30.7	15.6	0.1	21.8	0.5	0.1	0.4	0.8	2.3	0.6	0	19.0	2.2	1.2	0.2
Relative % Cyanobacteria Abundance	61.2	63.4	48.9	0.9	49.9	53.3	4.6	45.5	52.7	26.1	15.3	0	39.7	40.4	7.8	0.6

abundant in seawater samples at 7.6 and 17.8 % and represented 49.1% and 55.7% of Cyanobacteria sequences in samples W1 and W2, respectively (Table 2.6).

After three principal components, the model began to distinguish between individual coral samples. For example, negative separation along PC4 separated sample 3E (collected in 2009) from the remainder of the coral samples due to differences in Alphaproteobacteria composition. Phylotypes RKPE13, 22, 37, and 44 are phylotypes unique to the bacterial community of sample 3E and were low in abundance in the other coral communities sampled in 2006 (each phylotype was found at levels <0.02% in one other coral sequence library). This suggests collection year may be an important variable in octocoral-associated bacterial communities. The other Victory Reef samples 3A, 3C, and particularly 3D, separate from the remaining coral samples in a positive direction along PC4 (Figure 2.6C). This separation was most strongly influenced by the variables RKPE6, 23, and 25. Phylotype RKPE6 forms a clade with RKPE39 and is most proximal to an uncultured sponge associated Alphaproteobacteria with low sequence identity (HQ726945.2, 91% sequence similarity). These $OTU_{0.03}$s represented 21.3 and 8.0 % of the bacterial communities from corals 3C and 3D respectively. Phylotype RKPE25 formed a unique clade with an uncultured *Muricea elongata* (octocoral)-associated Gammaproteobacterium (DQ91704.1, 93% sequence similarity) and contributed to 9.5% of the bacterial community of coral sample sample 3D (as well as samples 1C, 1D, 2A, 2B, 3A/B, 3C, 4A and 4B at an abundance of 1% or less). Phylotype RKPE23 formed a clade with RKPE223, RKPE250 and was most closely related to an uncultured *G. ventalina* clone Gven_K23 (GU118518.1; 98% sequence similarity) and contributed predominantly to the bacterial community of samples 1E and 3A with an abundance of 83.2% and 23% respectively; however it was also found at a much lower abundance in coral samples 2B (3.3%), 3B (1.8%), 3C (0.8%)

Table 2.6: Population abundance of "*Prochlorococcus marinus*" clade phylotypes as an individual percent abundance, total percent abundance and relative percent abundance to total Cyanobacteria community of individual coral and water samples. Bold type indicates phylotypes that had the most influence on the PCA model.

PHYLOTYPE	1A	1B	1C	1D	2A	2B	3A	3B	3C	3D	3E	4A	4B	4C	W1	W2
RKPE9	**0**	**0**	**0**	0.02	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**4.9**	**10.6**
RKPE16	**0**	**0**	**0**	**0**	**0**	**0**	0.01	0.03	**0**	**0**	**0**	**0**	**0**	**0**	**2.7**	**7.2**
Total % Abundance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.6	17.8
Relative % Cyanobacteria Abundance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	49.1	55.7

and 3D (0.6%). Taxonomically, this clade may represent a novel phylum as it is most closely related to *Mycoplasma alkalescens* (80% sequence identity, NR025984.1) in the phylum Tenericutes. This phylotype was only detected in corals and was not found in surrounding seawater.

Ecological significance of RKPE1-related phylotypes

RKPE1-related phylotypes share an ancestral lineage with *Endozoicimonas elysicola* (NR_041264.1) and *Spongiobacter nickelotolerans* (AB205011.1) strains (91 and 93% similarity respectively). Based on 16S rRNA gene-based phylogenies (data not shown), it is likely that the two names may be synonymous; however, detailed polyphasic taxonomic characterization comparing cultured representatives from each genus is necessary to accurately delineate the two genera. *Spongiobacter/Endozoicomonas*-related phylotypes (SEPs) dominated the bacterial communities of *P. elisabethae*-associated bacterial sequence libraries 3A/B, 2B, and 4C (57.5, 71.9, 55.6, and 52.4%, respectively), were present in all but three coral sequence libraries, and were not detected in seawater libraries (Table 2.3). Based on low sequence similarity scores and nucleotide substitution distance within the phylogenetic tree to that of the related strains of *S. nickelotolerans* and *E. elysicola*, it is proposed that the related SEP phylotypes from *P. elisabethae* represent a novel genus of bacteria within the *Hahellaceae*. Although a functional role of bacteria from the *Hahellaceae* has yet to be determined, the repeated occurrence and dominance of related sequences within the bacterial community from healthy *P. elisabethae* specimens suggests a potential probiotic role.

SEP-related sequences have been identified previously from other healthy alcyonaceans, including *Sinularia* sp. (FJ654584.1, unpublished), *Cryogorgia koolsae* (HM173232.1, Gray *et al*., 2011), *Erythropodium caribaeorum* (DQ889928.1, unpublished), *Muricea elongata*

(DQ917877.1, unpublished), and the Mediterranean octocoral *Eunicella cavolini* (JQ691564.1, unpublished). Additionally, SEPs have been detected in healthy tissues of marine sponges (Kennedy *et al*., 2008; Webster *et al*., 2008), reef-building corals (Bourne *et al*., 2008; Yang *et al*., 2010) and in cultured toxic and nontoxic dinoflagellates (Hold and Moore, 2001). Two studies have demonstrated a specific association of SEPs with healthy specimens of the Great Barrier Reef coral *Acropora millepora.* For example, Bourne *et al*. (2008) observed a decrease in SEP prevalence in clone libraries concomitant with a loss of the dinoflagellate *Symbiodinium* sp. during a coral bleaching event and a return to dominance following reacquisition of the dinoflagellate after the bleaching event. The delicate balance between bacteria and endosymbiotic zooxanthellae may be explained by the fact that while *Symbiodinium* spp. are predominantly responsible for producing dimethylsulfoniopropionate (DMSP), marine bacteria are capable of degrading DMSP to dimethyl sulfide (DMS) (Raina *et al*., 2009). This dynamic may explain why some bacterial communities in acroporid corals may disappear from the coral community following the expulsion of algal symbionts; furthermore, this suggests SEPs may be important indicators of coral health owing to their sensitivity to disturbance. Collectively, these observations suggest that utilization of organic sulfur compounds may strongly influence the structure of coral-associated bacterial communities. Indeed, all cultured SEPs have been isolated from marine invertebrates which have a close association with algal producers of DMSP, either in the form of symbiotic relationships (corals – dinoflagellates, sponges – Cyanobacteria) or dietary consumption (sea slug – dietary algae; Van Alstyne, 2008). To experimentally establish the role of DMSP content as a driver of octocoral bacterial community structure, future efforts could focus on determining if a link exists between DMSP content and the presence of specific phylotypes.

As mentioned previously, the role that RKPE1-related phylotypes play in octocorals is unclear, as uncultured phylotypes reveal little about their biological roles in the holobiont. Although further research would be required, it is possible that, based on the abundance and dominance of these phylotypes in *P. elisabethae* within and between reefs throughout the Bahamas, these bacteria may be involved in natural product (pseudopterosin) biosynthesis as hypothesized in other investigations of bacteria associated with marine invertebrates (Pérez-Matos *et al*., 2007). Moreover, the predominance of this phylotype in *P. elisabethae* bacterial communities indicates this phylotype has a selective advantage or predisposition to utilize the coral as a substrate to support the growth and overall fitness of the bacterium. Conversely, the ubiquitous presence of RKPE1-related Gammaproteobacteria phylotypes may simply be a function of abundance, and may not play an ecologically significant role in the host. For instance, fluorescence *in situ* hybridization studies in the gastroderm of branching stony corals revealed large ovoid bacterial aggregates were consistently identified as Gammaproteobacteria (Ainsworth *et al*., 2006). Hence, it is possible that the observed abundance of RKPE1-related Gammaproteobacteria phylotypes in *P. elisabethae* may be due to the formation of large aggregates consisting of closely related colonies in the coral holobiont. However, the absence of RKPE1 phylotypes in the water column indicates these bacteria may be true endosymbionts which have specifically co-evolved with their marine invertebrate hosts.

Flavobacteria

Representatives of the *Aquimarina* spp. phylotype clade (*e.g.* RKPE2, Figure 2.5) were found on coral samples from both Eleuthera Island (Sample 1C, 30.3% abundance) and Victory Reef (Sample 3C, 41.3% abundance; Sample 3E, 10.5% abundance). Their discovery from samples across different geographical locations, as well as demonstrating the ability to establish

a dominant bacterial community on at least some coral samples suggests these bacteria may have a specific association with *P. elisabethae*. *Aquimarina* is a recently established genus within the Flavobacteriacea (Nedashkovskaya *et al*., 2005) representing aerobic, heterotrophic, gliding, Gram-negative, marine bacteria that produce flexirubin-type non-diffusible pigments. The 16S rDNA sequence of RKPE2, the dominant representative phylotype of this clade within *P. elisabethae*-associated bacterial communities (Table 2.4), is most similar to the type strain *Aquimarina muelleri* (93%). To date, the genus contains eight taxa: *A. muelleri*, *A. addita*, *A. brevivitae*, *A. intermedia*, *A. latercula*, *A. macrocephali*, *A. salinaria*, and *A. spongiae*; all are widely distributed in various marine environments and isolated from substrates ranging from seawater and sediment to a sea urchin and a sponge. The *P. elisabethae* phylotypes form two distinct clusters in the *Aquimarina* clade suggesting they may represent at least two new *Aquimarina* species (Figure 2.5). Although the role of these organisms in *P. elisabethae* is unknown, *A. salinaria* has demonstrated algicidal activity in co-culture experiments (Chen *et al*., 2012); thus *P. elisabethae*-associated *Aquimarina* spp. may play a role in the preventing algal fouling or mediating interactions between the coral and its algal photosymbionts. As taxa of this genus can readily be cultured, future culturing of RKPE2 may be achievable and would allow for the testing of these hypotheses.

Cyanobacteria

Closely-related *Synechococcus* sp. and *Prochlorococcus* sp. $OTU_{0.03}$s were consistently abundant as members among the Cyanobacterial pyrosequencing libraries and were shared between coral and water libraries (Table 2.5, 2.6). *Synechococcus* and *Prochlorococcus* are unicellular autotrophs which are ubiquitously distributed throughout the world's oceans and comprise a diverse group of Cyanobacteria, where phylogenetic and physiological data have

been used to subdivide the group into 18 distinct clades. These are hypothesized to represent physiologically and ecologically distinct populations or ecotypes and whose distribution varies both spatially and temporally (Choi and Noh, 2009; Fuller *et al*., 2003; Rocap *et al*., 2002). The most abundant phylotype isolated from the coral samples was 99% similar by 16S rRNA gene sequencing to *Synechococcus* sp. strain KORDI-54, which belongs to *Synechococcus* clade VI (Choi and Noh, 2009) and represented a moderate proportion of the overall bacterial community from *P. elisabethae* samples 1A/B, 1C, 2A and 4B (Table 2.5). Similarly, *Prochlorococcus* sp. contributed to the Cyanobacteria community in water sequence libraries, yet were less abundant in corals (Table 2.6). Ecologically, *Synechococcus* sp. appear to form stable, long-term associations with a variety of invertebrates and produce amphiphilic siderophores to acquire iron from the surrounding environment (Ito and Butler, 2005). Furthermore, *Synechococcus* sp. and *Prochlorococcus* sp. co-exist with symbiotic dinoflagellates in the Caribbean hard coral *Montastraea cavernosa* and may play a role in nitrogen fixation, as conspecifics lacking these endosymbionts cannot fix nitrogen (Lesser *et al*., 2004, 2007). Since *Synechoccocus* sp. are globally distributed and abundant in seawater (Choi and Noh, 2009; Dinsdale *et al*., 2008), it is likely that *P. elisabethae* may be ingesting/obtaining these phylotypes from the surrounding environment. In addition, the high abundance of these phylotypes suggests that *Synechoccocus* sp. occupy a niche in the coral holobiont.

Alphaproteobacteria

Alphaproteobacteria have an extensive history of forming symbiotic relationships with other taxa (Bates *et al*., 2010; Jackson *et al*., 2006). Alphaproteobacteria are both diverse and ubiquitously distributed in both stony corals and some octocorals (Brück *et al*., 2007; Meron *et al*., 2011; Rohwer *et al*., 2001; Sweet *et al*., 2011). In contrast to the closely-related

Cyanobacteria and Gammaproteobacteria phylotypes obtained in this study, dominant RKPE $OTU_{0.03}$s in the class Alphaproteobacteria were represented by diverse phylotypes (Figure 2.5). This is in opposition to an acroporid study by Meron and colleagues, who demonstrated that Alphaproteobacteria were dominated by *Rhodobacteracea* (Meron *et al*., 2011). Furthermore, due to the low sequence identity of RKPE6 to reference sequences in GenBank (91% sequence identity to an uncultured sponge-associated bacteria; 85% sequence identity to *Terasakiella pusilla* (NR_024656.1), this suggests RKPE6 is a potentially novel bacterial order or family. Furthermore, some Alphaproteobacteria phylotypes from *P. elisabethae* showed sequence similarity to bacteria obtained from bleached corals and from polluted/wastewater (Figure 2.5), which may imply that the health of *P. elisabethae* requires monitoring.

Unclassified bacteria

A candidate bacterial phylum based on RKPE23-related phylotypes (RKPE23, 223, and 250) was identified from *P. elisabethae* which were devoid in water-associated bacterial sequence libraries. Interestingly, these phylotypes were particularly abundant in gDNA derived pyrosequencing libraries (sample 1E, 82.8%; sample 3A, 23.0%) and were less abundant in PCR amplicon libraries (sample 3B, 1.2%). These phylotypes were also responsible for driving the separation of *P. elisabethae* associated bacterial libraries along PC1 in the PCA biplot (Figure 2.6A). Phylogenetic identification of RKPE23 with the nearest type strain revealed RKPE23 was most similar to a *Mycoplasma alkalescens* strain in the phylum Tenericutes (80% sequence identity); however, the RKPE23 phylotype matched at 98% sequence similarity to an uncultured bacterium clone obtained from the Caribbean octocoral *G. ventalina* (clone Gven_K23). Although this phylotype thus-far appears to be restricted to octocorals based on RDP probe matching, it is difficult to define the taxonomic significance of this phylotype due to its highly

distant relationship to *M. alkalescens*. However, the conservation of shared bacteria between different octocorals provides evidence which supports the hypothesis that alcyonaceans share common bacterial symbionts.

Detection of potentially novel bacterial phylum from *P. elisabethae*

Due to the novelty of the RKPE23 phylotype, a taxa-specific primer was designed and amplicons were constructed for each coral library. Alignment of RKPE23 and RKPE223 with *G. ventalina* clones revealed a unique 10 bp sequence insert that was not detected in another *G. ventalina* clone (Gven_G10) or *E. coli* (Figure 2.7). Furthermore, despite the fact that the RDP database contains almost 2 million 16S rRNA gene sequences, this 10 bp sequence was only retrieved from the 16S rRNA gene sequence for RKPE23 and *G. ventalina* clone Gven_K23. Consequently, a 25 bp primer was designed to amplify this unique fragment from corals (Figure 2.7). Amplification was successful for 10 of 13 corals based on the detection of an approximately 200 bp band by 1% AGE (Table 2.7; Figure 2.8), indicating that this phylotype was not a sequence artifact from bTEFAP library preparations and revealing that this phylotype appears to be conserved across *P. elisabethae* throughout the Bahamas. Furthermore, the absence of evidence of this band from the negative control (*E. coli*) supports that RKPE23-related phylotypes are specific to octocorals.

Comparison of *P. elisabethae* and water-associated bacterial communities

To compare coral and water-associated bacterial communities, water sequence libraries (W1 and W2) were clustered with coral sequence library 3E. The water sample used to prepare the W2 sequence library was collected adjacent to sample 3E, while the W1 sequence library was prepared from a water sample collected the same day yet from 5 km away. In total, 2,047

```
RKPE223              A-TGTGTAAAGACATATGTTTT-ATTATTAAAGGATCGTTTTGGTCCG-CGCAG--GGAT
Gven_K23             A-TGTGTAAAGACATATGTTTTTATTATTAAAGGATCGTTTTGGTCCG-CGCAG--GGAT
RKPE23               A-TGTGTAAAGACATATGTTTTTATTATTAAAGGACCGTTTTGGTCCGGCGCAG--GGAT
Gven_G10             ACCTTCTAAGGAAGAAAGC----------AGGGGATC-TTCGGACCTTGCGCTATCGGAT
Ecoli16S         179 AACGTCGCAAGACCAAAGA----------GGGGGACC-TTCGGGCCTCTTGCCATCGGAT 227
                     *   *   * **   * *             *** * **  *  *    **    ****
```

RKPE23 (Gven_K23) Designed Primer:
5' – AAAACGATCCTTTAATAATAAAAAC-3'

Figure 2.7: DNA alignment of potentially novel RKPE phylotype sequences from *P. elisabethae*. Included in the alignment is the closely related clone Gven_K23 (GU118339.1) along with Gven_G10 (GU118518.1) and *E. coli* (J01695.2). The alignment positions are relative to *E. coli* shown from positions 179 – 227. The designed primer containing the unique 10 bp sequence is highlighted below.

Table 2.7: RKPE23 phylotypes detected in coral libraries

Site	Coral sequence library	Gven K23/RKPE23 detected
Eleuthera Reef	1A	Yes
Eleuthera Reef	1B	Yes
Eleuthera Reef	1C	No
Eleuthera Reef	1D	Yes
Eleuthera Reef	1E	Yes
Sweetings Cay	2A	Yes
Sweetings Cay	2B	Yes
Victory Reef	3A/3B	Yes/Yes
Victory Reef	3C	Yes
Victory Reef	3D	Yes
Bimini	3E	No
Petersons Reef	4A	Yes
Petersons Reef	4B	No
Petersons Reef	4C	Yes

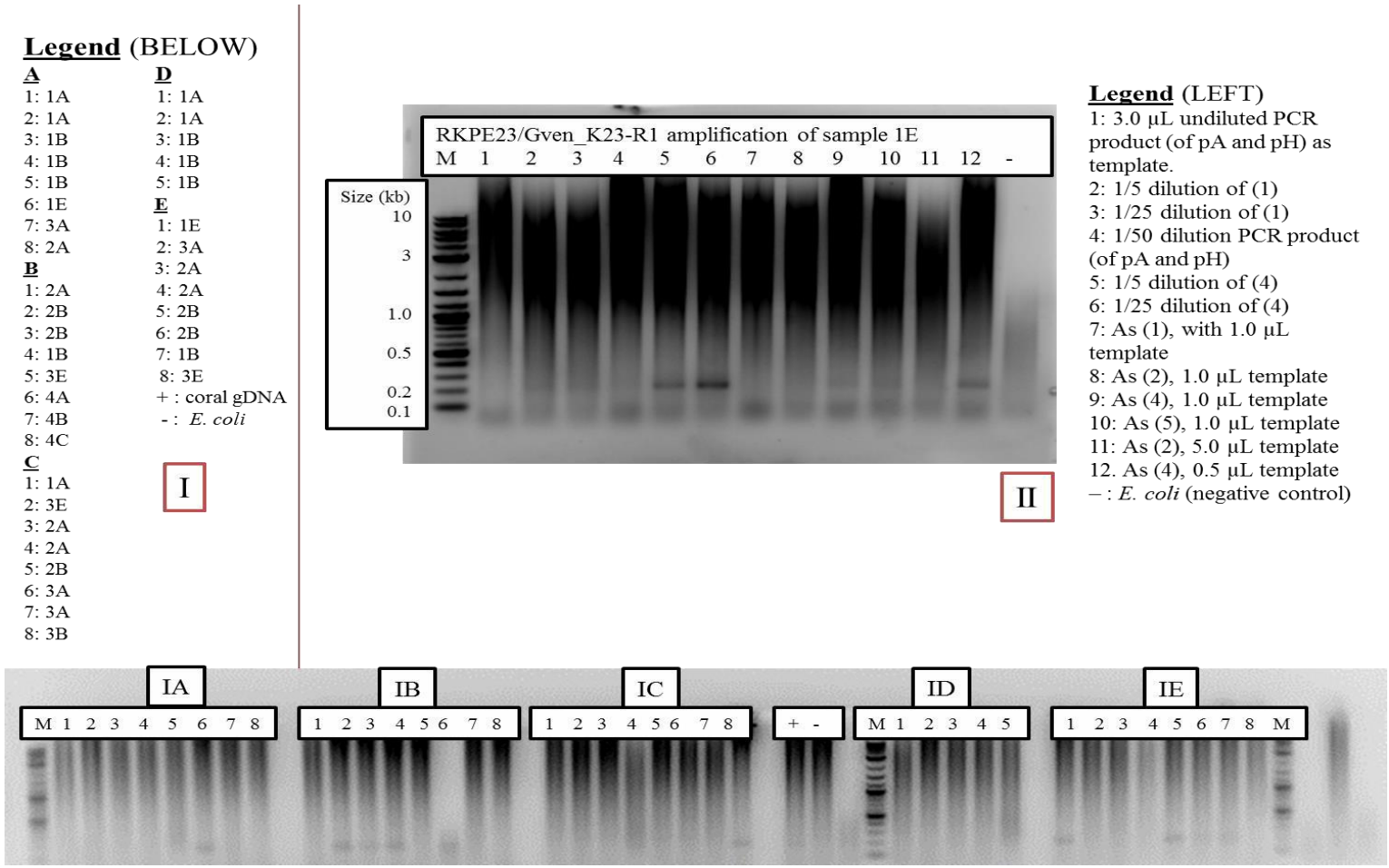


Figure 2.8: RKPE23/Gven_K23-R1 screening results from *P. elisabethae*. A) Amplification results of *P. elisabethae* sample libraries, B) Amplification results of sample 1E, which contained the greatest abundance of RKPE23 (83.8%). Shown is sample 3E amplified using the conditions listed in the legend; "M" refers to molecular marker (2-log ladder).

$OTU_{0.03}$s were present in the three sequence libraries. Phylotypes from sample 3E outnumbered both water library phylotypes at $OTU_{0.03}$, generating an additional 107 $OTU_{0.03}$s not detected in water. Based on a presence/absence matrix (data not shown), 52.0% of $OTU_{0.03}$s were unique to sample 3E, 46.8% were unique to W1/W2, and only 1.1% were conserved between water and sample 3E sequence libraries. Dominant coral phylotypes were not found in seawater libraries and *vice versa*. One notable exception to this was observed among Cyanobacteria-affiliated sequences in which a variety of related *Synechococcus* sp. and *Prochlorococcus marinus* phylotypes overlapped between coral and seawater sequence libraries (Table 2.6). Furthermore, Alphaproteobacteria (*Pelagibacter* sp., Figure 2.5) were present in both the water column and corals, suggesting that *P. elisabethae* may be actively selecting (or possibly consuming) a minimal number of microbes from the surrounding environment. In general, however, the abundance of unique *P. elisabethae*-associated phylotypes and the lack of conservation between coral and water-associated bacterial communities suggest the surrounding water exerts little influence on shaping the bacterial community of *P. elisabethae* and supports previous observations that healthy corals maintain a unique microbial community from the surrounding water column (Bourne and Munn, 2005; Kvennefors *et al*., 2010; Rohwer *et al*., 2001; Sunagawa *et al*., 2010; Sweet *et al*., 2011).

Evaluation of sequencing methodology

As bTEFAP is a relatively novel method for assessing coral-associated bacterial taxonomy, the reproducibility, accuracy, and suitability of the method was tested by comparing A) replicate 16S rRNA gene pyrosequences (sequence libraries 1A and 1B) as a template for bTEFAP which were submitted independently, B) a gDNA sample in conjunction with its PCR-derived amplicon (sample 3A/3B) submitted in the same pyrosequencing run, and C) bTEFAP

sequences to dominant DGGE band sequences. The class-level community profiles of 1A and 1B were strikingly similar with only moderate differences observed in the abundance of Alphaproteobacteria and Cyanobacteria (Figure 2.3). Similarly, the class-level community structure deduced from sequence libraries 3A and 3B were generally similar, but differed in the relative portion of Gammaproteobacteria and unclassified bacterial sequences (Figure 2.3), confirming that nested PCR did not introduce significant bias into the analysis. This was also reflected in the PCA model, as PCR amplicon replicates (1A and 1B) and gDNA/PCR amplicons (3A and 3B) contained a similar overall bacterial community and separated along all principal components in spatial proximity to one another respectively based on species-level phylotypes (Figure 2.6). These results suggest that the variation between experiments is relatively low and that large observed differences in bacterial community structure in *P. elisabethae* are not likely due to methodological variation. However, there were a few notable differences between the gDNA and PCR amplicons. Comparison of bacterial communities generated from gDNA and PCR amplicon pyrosequences showed that PCR amplicons yielded a greater number of pyrosequencing reads (868 for gDNA (3A) and 2387 for PCR amplicon (3B) pyrosequences respectively), higher bacterial diversity, and less unclassified bacteria (Table 2.1). In part, the differences observed between these samples may be due to the low number of sequences (868) obtained using gDNA (3A) as template. The major difference was highlighted at the species level based on the PCA model (PC4) due to differences in the relative abundance of the RKPE23 phlyotype, while sample 1E (gDNA) was removed as an outlier from the PCA biplot due to the overrepresentation of this phylotype (Figure 2.6A). It is unclear why these phylotypes were detected in much greater abundance in the gDNA sequence libraries compared to PCR amplicon libraries. However, the dominance of unclassified bacteria in gDNA libraries suggests that

gDNA pyrosequencing is superior in uncovering higher amounts of rare and unclassified taxa which may become reduced over sequential rounds of PCR amplification. Consequently, the use of gDNA templates for bTEFAP is recommended to assess bacterial diversity in octocorals. This conclusion is drawn from one gDNA pyrosequencing dataset and may not be representative in all cases. Taken together, the results suggest that bTEFAP is a reproducible and reliable method for characterizing bacterial communities and diversity in a healthy alcyonacean octocoral.

Comparison of bacterial communities by pyrosequencing and DGGE

Sequences obtained for excised DGGE bands were directly compared to bTEFAP sequences as a result of amplifying the same region on the 16S rRNA gene. In total, eight dominant bands were sequenced from six representative corals (Figure 2.9). Interestingly, dominant $OTU_{0.03}$s in bTEFAP did not necessarily reflect the most dominant DGGE retrieved band sequence (Table 2.8) and did not reflect the numerical abundance of Alphaproteobacteria or Gammaproteobacteria as seen in bTEFAP sequence libraries. In several cases, however, DGGE band sequences matched those of dominant RKPE phylotypes (Table 2.8). The most frequently retrieved band (*e.g.* in five of the eight bands) was most closely related to *Synechococcus* sp. (Bands 3, 5, and 7; Table 2.8), demonstrating that DGGE and bTEFAP are complementary tools for identifying the abundance of Cyanobacteria in an octocoral. In addition, DGGE confirmed the presence of the bTEFAP-generated RKPE23 related phylotype (Band 1) from sample 3A and its PCR equivalent sample 3B (Table 2.8), classified as relating to the uncultured *G. ventalina* Gven_K23 phylotype, which was an abundant $OTU_{0.03}$ in bTEFAP libraries (17.6% in sample 3A and 1.2% in sample 3B). DGGE band 2 confirmed the presence of the bTEFAP phylotype RKPE6, representing an uncultured alphaproteobacterium (Table 2.8) along with two *Mycobacterium caprae* – related phylotypes from sample 1C and sample 2A (bands 4 and 6).

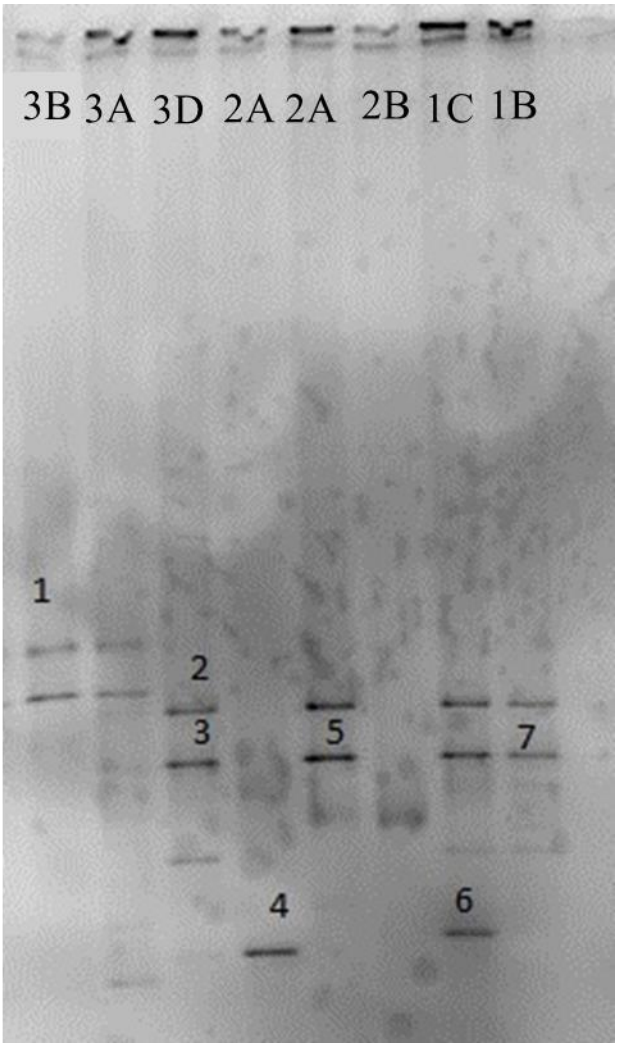


Figure 2.9: DGGE polyacrylamide gel displaying 16S rRNA gene sequence bands sequenced from representative *P. elisabethae* corals. Band sequence identities are indicated in Table 2.9. Lanes with no band numbers and visible bands without band numbers indicate no sequences could be obtained due to ambiguous sequence data.

Table 2.8: Comparison of DGGE band sequences to bTEFAP sequences from selected corals. NA: Band not available on the gel in Figure 2.9. DGGE band numbers correspond to labels in Figure 2.9.

Sequence library	DGGE band no.	Base pair similarity to bTEFAP	Closest BLASTn similarity (GenBank Accession No.; percent identity of match)	Sequence abundance in coral bTEFAP library	Most dominant $OTU_{0.03}$ in bTEFAP library? (Table 3)
1B	7	402/402, 100%	*Synechococcus* sp. KORDI-54 (FJ497735.1; 100%) (Cyanobacteria)	13.6%	Yes
1C	6	408/418, 96%	*Mycobacterium caprae* (NR_028879.1; 95%) (Actinobacteria)	<1% of total library	No
2A	5	402/402, 100%	*Synechococcus* sp. KORDI-54 (FJ497735.1; 100%) (Cyanobacteria)	23.7%	No (2nd most abundant)
2A	4	386/388, 99%	*Mycobacterium caprae* (NR_028879.1; 98%) (Actinobacteria)	<1% of total library	No
3A and 3B	1	424/425, 99%	Uncultured bacterium clone Gven_K23 (GU118339.1; 99%) (Unknown phylum)	24.2%	No (2nd most abundant in Sample 3A)
3C	NA	402/402, 100%	(*Synechococcus* sp. KORDI-54 (FJ497735.1; 97%) (Cyanobacteria)	1.49%	No
3D	2	407/419, 97%	Uncultured sponge Alphaproteobacterium (HQ726945.1, 92%) (Alphaproteobacteria); Uncultured Proteobacterium clone RKPE6 (JN411722.1; 99%)	7.7%	No (2nd most abundant)
3D	3	405/406, 99%	*Synechococcus* sp. KORDI-54 (FJ497735.1; 100%) (Cyanobacteria)	1.14%	No

Only a few bands were generated from the use of DGGE, which may be a function of experimental difficulties. For example, at 507 bp, the modified primer pair 27f-GC and P2 (Muyzer *et al*., 1993) exceeded the 500 bp limit of resolution for DGGE (Muyzer and Smalla, 1998). In addition, fewer bands could be sequenced. In this case, despite numerous attempts at purification, more than one band was present on the polyacrylamide gel (data not shown). As a consequence, attempts to sequence the excised bands produced highly ambiguous 16S rDNA sequences. As a result of the inherent difficulties of DGGE and in combination with the simplicity and efficacy of bTEFAP, bTEFAP is recommended as a superior methodology at inferring coral-associated bacterial diversity.

Screening *P. elisabethae* for the presence of associated zooxanthellae

Zooxanthellae from five selected *P. elisabethae* samples were a match to other stony and octocoral-associated zooxanthellae based on sequence identity. Here, zooxanthellae band sequences exclusively represented *Symbiodinium* clade B (Table 2.9), a trend which has been reported previously for *P. elisabethae* (Santos *et al*., 2003; 2004). Interestingly, the octocorals *P. elisabethae* and *G. ventalina* host phylogenetically similar *Symbiodinium* sp. (Santos *et al*., 2004) and as mentioned previously, share some dominant bacteria (*e.g*. RKPE1 and RKPE23-related phylotypes). Octocoral taxonomists state that the two genera (*Pseudopterogorgia* spp. and *Gorgonia* spp.) are more closely related than previously believed based on mitochondrial DNA sequencing (McFadden *et al*., 2006; 2010; Sanchez *et al*., 2003). Consequently, the genetic similarities and the similarity of algal symbionts between *G. ventalina* and *P. elisabethae* may be one factor which explains why these corals are hosting similar dominant bacteria. Given that octocoral cell signalling controls dinoflagellate populations (Koike *et al*., 2004), it is perhaps not implausible to suggest that the dinoflagellate community may be involved in shaping octocoral-

Table 2.9: Zooxanthellae band sequence identities from selected *P. elisabethae* samples.

Sequence library	Nearest BLAST sequence identity	Sequence length (bp)	E	Percent maximum sequence identity (%)
1A	*Plexaura kuna* (octocoral, Panama)-associated *Symbiodinium* sp. clade B1 (JN558057.1)	614	0	99
2A	*Plexaura kuna* (octocoral, Panama)-associated *Symbiodinium* sp. clade B1 (JN558059.1)	458	0	99
2B	*Diploria strigosa* (stony coral, Bermuda)-associated *Symbiodinium* sp. clade B (AY074972.1)	602	0	100
4A	*Plexaura kuna* (octocoral, Panama)-associated *Symbiodinium* sp. Clade B1 (JN558057.1)	601	0	99
4C	*Montastrae annularis* (stony coral, Curacao)-associated *Symbiodinium* sp. Clade B1 (DQ200711.1)	279	0	98

associated bacterial communities and thus may play a role in conserving at least some members of the bacterial community in *P. elisabethae* and *G. ventalina*.

The bacterial community of *P. elisabethae* is not conserved within and between reefs in the Bahamas

Both community analysis at the class level (Figure 2.3) and PCA at the species level (Figure 2.6) revealed that the *P.elisabethae*-associated bacterial community was not highly conserved among individuals within a site; rather, specific dominant $OTU_{0.03}$s within the bacterial community were shared by coral samples from different sites (Figure 2.6B – C), suggesting that bacterial communities are diverse and distinct both locally and throughout the Bahamas. In contrast to the present research, however, many studies report that corals host a stable and species-specific relationship with bacteria (Rohwer *et al*., 2001, 2002; Rosenberg *et al*., 2007). However, debate surrounding the specificity of bacterial relationships to cnidarians exists owing to contradictory evidence which suggest bacterial diversity is variable over time (Lee *et al*., 2009), site (Gray *et al*., 2011; Kellogg *et al*., 2009; Kvennefors *et al*., 2010; Penn *et al*., 2006), species (Lee *et al*., 2011; Neulinger *et al*., 2009), life stage (Littman *et al*., 2009a), and spatial location within the cnidarian holobiont (Bourne and Munn, 2005; Qian *et al*., 2006; Schöttner *et al*., 2009; Sweet *et al*., 2011; Wichels *et al*., 2006). For example, acroporids collected in triplicate from two locations in the Great Barrier Reef revealed that acroporid bacterial communities grouped according to location rather than species (Littman *et al*., 2009b). Additionally, bacterial communities of triplicate stony coral libraries not only showed bacterial variation within the same environment, but also across locations and by colour morph (Neulinger *et al*., 2008). Furthermore, clone libraries from bacteria associated with deep-sea alcyonaceans collected at different locations in the Aleutian Islands showed no clear pattern of a conserved

microbial consortium, with two clone libraries dominated by Tenericutes and Alphaproteobacteria, while other coral-associated bacterial libraries were more diverse (Gray *et al*., 2011). Furthermore, the authors found that bacteria were more similar between congenera than between conspecifics. This trend in bacterial community variation also extends to other eukaryotes. For example, the bacterial community in the plant phyllosphere on cottonwood trees was highly variable throughout the growing season (Redford and Fierer, 2009). Interestingly, each of the samples collected from the same tree on different dates harboured a distinct bacterial community; furthermore, the variability in bacterial communities between different trees sampled on the same date was less than the variability in a single tree over time. The authors suggested that environmental perturbations may prevent the stabilization of the bacterial community over time. Therefore, it is possible that local environmental conditions may have impacted the bacterial community in *P. elisabethae* at least in part.

Factors influencing microbial diversity analysis

Several factors may affect the determination of microbial communities. For example, the quality and completeness of DNA extractions are regarded as the most influential factor in shaping the diversity and community of 16S rRNA gene sequences obtained from invertebrate hosts (Feinstein *et al*., 2009, Pan *et al*., 2010). In particular, alcyonacean octocorals are notoriously difficult to obtain high quality DNA which enables PCR amplification, a factor which is most likely due to the incomplete lysing of bacterial cells and the co-extraction with phenolic compounds (Gray *et al*., 2011; Sunagawa *et al*., 2010). These difficulties may explain the lack of investigations characterizing octocoral-associated bacteria. In addition, lysis efficiency of bacterial cells varies depending on the targeted group. The highly rigorous conditions required for lysis of Gram-positive bacteria, including bead beating, causes shearing

of nucleic acids in Gram-negative bacteria, leading to problems in downstream PCR amplification (Kirk *et al*., 2004; von Witzingerode *et al*., 1997). PCR amplification also significantly impacts measures of microbiological diversity. For example, Wu *et al*. (2010) showed polymerase choice in PCR amplification had the most significant effect on the analysis of microbial diversity with next generation sequencing methods, where a high fidelity polymerase generated less PCR artifacts and determined significantly different community structures based on efficiency in amplifying abundant sequences. Hence, the results may have been negatively impacted by the use of EconoTaq® DNA polymerase in PCR amplification. Authors have stressed that compounds co-extracted from environmental sources, mainly phenolic compounds (including humic substances), strongly inhibit *Taq* polymerases (von Witzingerode *et al*., 1997) and denatures biological molecules (Young *et al*., 1993). Furthermore, classifying bacterial species at 97% sequence similarity and based on partial sequences (<500 bp) can be misleading in phylogenetic placement (Hugenholtz *et al*., 1998). Consequently, it has recently been proposed by Kim *et al*. (2011) that clustering 16S rDNA sequences at the V1 – V3 region at $OTU_{0.04}$ provides a more accurate estimate of species diversity. The choice to cluster at the conventional species level (97% sequence similarity, Cohan, 2002) in this investigation may explain the abundance of closely-related phylotypes obtained and possibly suggests that diversity measurements may be overestimated.

Finally, the results demonstrate that *P. elisabethae* contains an abundance of bacterial phylotypes with the likely ability to produce natural products. For example, marine-derived Cyanobacteria continue to be an important source of bioactive metabolites and have yielded a greater amount of compounds than their terrestrial counterparts (Gulder and Moore, 2009; Williams, 2009). In addition, Flavobacteria produce metabolites which have antitumor activity

and Bacillales produce compounds with antimicrobial properties (de Carvalho and Fernandes, 2010), while Actinomycetales account for up to 45% of promising new bioactive compounds (Williams, 2009). In the present study, five of six dominant Actinobacteria phylotypes were exclusive to *P. elisabethae* libraries (5% abundance) and were not detected in surrounding water samples. These results suggest that *P. elisabethae*-associated bacteria may be an excellent resource to investigate for drug discovery. This will be discussed in detail in subsequent chapters.

CONCLUSIONS, SIGNIFICANCE, AND FUTURE AIMS

This study represents the first in-depth analysis on the geographic variability of the bacterial communities in the octocoral *Pseudopterogorgia elisabethae* and has demonstrated the suitability of bTEFAP for inferring octocoral-associated bacterial taxonomy. This study has also identified species-level phylotypes which are the main contributors to the differences among coral and water-associated bacterial sequence libraries. This level of detail has not been previously addressed to our knowledge. Based on the analysis of the *P. elisabethae* bacterial community, the evidence presented herein suggests the coral exerts little influence on shaping its bacterial community. However, the results also suggest that the octocoral provides a specialized habitat for bacteria which are distinct from those of the surrounding seawater. Specifically, the results demonstrate that Alphaproteobacteria were more diverse than Flavobacteria, Gammaproteobacteria, and Cyanobacteria. *P. elisabethae* also harbours potentially novel species and genera, while a new candidate bacterial phylum was also identified. If strains of the previously mentioned novel species, genera, or phyla could be isolated, cultivation studies of coral-associated bacteria may help to reveal further insight as to why these bacteria were predominant within the bacterial community of *P. elisabethae.*

This study has contributed to the general scientific understanding of bacteria associated with octocorals, which still remains a little-studied topic. Future investigations may consider studying additional alcyonaceans in order to determine if the lack of bacterial conservation between individuals in *P. elisabethae* can be extended to other octocorals. The impact of this research to the scientific community has been in the form of identifying potentially novel bacterial species, genera, and a candidate phylum. Given the well-established link between

biological diversity and natural product diversity, and based on the novelty of some of its associated bacteria, *P. elisabethae* is an excellent candidate for drug discovery programs. However, based on the evidence presented herein, bioprospecting strategies should consider sampling multiple individuals from *P. elisabethae* within and between reefs to fully realize the microbial diversity harboured by this commercially important octocoral. Following successful cultivation, *P. elisabethae*-associated bacteria may provide a sustainable source of new and useful natural products for biomedical applications.

Another interesting conclusion from this study is that *Pseudomonas aeruginosa* may not be the sole biosynthetic origin of the pseudopterosins as previously hypothesized (Bunyajetpong *et al*., 2011; Bunyajetpong, 2011). One would presume that a true pseudopterosin-producing bacterium would be ubiquitously distributed in all *P. elisabethae* specimens as has been shown in other marine invertebrates (Perez-Matos *et al*., 2007). Given that *Pseudomonas* spp. were not consistently recovered in corals throughout the Bahamas, one might reasonably conclude that pseudopterosin production may be the result of the acquisition of a plasmid containing the pseudopterosin biosynthetic gene cluster from *P. aeruginosa*. Although this study indicated there is significant variability in the bacterial community, *Ruegeria* sp. (RKPE12 phylotype) were consistently detected across all PCR-generarted coral libraries, while RKPE1 phylotypes (91% sequence similarity to *Endozoicomonas* sp.) were particularly dominant in select sequence libraries. Since *Ruegeria mobilis* isolated from *Symbiodinium* sp. within two reef-building corals has previously been demonstrated to horizontally transfer genetic material between bacterial taxa (Young, 2011), in addition to the ubiquitous distribution across *P. elisabethae* samples in the Caribbean, it is possible that *Ruegeria* sp. may be an alternative source of the pseudopterosins. A future experiment may consider screening coral-associated *Ruegeria* sp. for the presence of

terpene-natural product biosynthetic gene clusters to determine whether *Ruegeria* sp. play a role in pseudopterosin biosynthesis.

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CHAPTER III

INVESTIGATION OF THE CULTIVABLE BACTERIAL COMMUNITY FROM *PSEUDOPTEROGORGIA ELISABETHAE*

INTRODUCTION

Corals are host to a diverse consortium of microorganisms, including zooxanthellae, bacteria, and fungi (Goulet and Coffroth, 2004; Sunagawa *et al*., 2010; Wang *et al*., 2011). In the past, culture-based studies dominated our understanding of coral-associated microbiology (Ducklow and Mitchell, 1979; reviewed in Rosenberg *et al*., 2007), however, owing to the limitations associated with cultivation-based techniques (Engelbrekson *et al*., 2010; Joint *et al*., 2010; reviewed in Uthicke and McGuire, 2007), culture-independent investigations of coral-associated bacteria have taken precedence in the literature (Rohwer *et al*., 2001; Rosenberg *et al*., 2007; Sunagawa *et al*., 2010). While it is important to acknowledge that culture-based studies underestimate bacterial diversity (Engelbrekston *et al*., 2010), Bergey's Manual of Systematic Bacteriology states that an organism cannot be classified unless its morphological, cultural, physiological and pathogenic characteristics have been described via study of pure and viable cultures (Breed and Smithh, 1957). Furthermore, cultivation provides a resource to practically assess the biomedical capabilities of bacterial metabolites, as cultivation is an essential prerequisite for biodiscovery (Galkiewicz *et al*., 2011). However, although a wealth of literature exists reporting culturable bacteria from scleractinian corals (Ben-Dov *et al*., 2009; Nithyanand and Pandian, 2009; Nithyanand *et al*., 2011), little attention has been focused on the isolation and cultivation of bacteria from commercially important octocorals.

Previously, pyrosequencing analysis of bacterial communities associated with the octocoral *P. elisabethae* revealed that the coral harbours a diverse bacterial community (refer to Chapter II). Given the commercial significance of *P. elisabethae* (Roussis *et al*., 1990) and evidence that associated bacteria may be the true source natural products previously ascribed to the host invertebrate (Bunyajetpong *et al*., 2011; Bunyajetpong, 2011; Davidson *et al*., 2001; Piel *et al*., 2002; 2004), characterizing the prokaryotic community associated with this important octocoral can provide a resource with which to discover new sources of natural products. Additionally, comparing the cultivable community to the culture-independent community will contribute to the scientific understanding of alcyonacean-associated bacterial communities and will ultimately enhance our ability to design strategies with which to isolate natural product producing bacteria. The research goals in this study are:

1) To characterize the cultivable diversity of bacteria from *P. elisabethae* and to maximize the diversity of recovered isolates through the use of a diverse selection of isolation media and the particle filtration technique (PFT),

2) To determine the success of the selected isolation strategy at culturing a representative selection of the *P. elisabethae* microbiome via comparison of the metagenomic 16S rDNA sequence libraries to cultured isolates,

3) To assess the ability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a tool to de-replicate environmental isolates, and

4) To identify the salinity requirements of coral-associated bacteria and to test their susceptibility to pseudopterosin G, an antimicrobial diterpene from *P. elisabethae*.

As less than 1% of microorganisms can be cultured using standard plating procedures, techniques which favour the detection of rare species have been devised to overcome the limits of cultivation (Hawksworth and Rossman, 1997; Kaeberlein *et al*., 2002). Specifically, the PFT has been successfully implemented as a tool to detect slow growing fungi; ultimately, this technique has resulted in increasing the number of fungal species by ten times more than other methods (Hawksworth and Rossman, 1997). Here, the PFT is introduced in an attempt to isolate potential symbiotic bacteria, where it is hypothesized that screen filters will allow access to tissue-associated bacteria by enabling bacteria to physically grow in the presence of coral fragments. It is hypothesized that the highly diverse media conditions in conjunction with the particle filtration technique will result in a highly diverse culturable bacterial community from *P. elisabethae*. The goal in testing for salinity requirements was to add to the scientific understanding of those bacteria isolated from marine invertebrates which are dependent on seawater for growth. Although evidence suggests clinical Gram-positive bacteria are inhibited by pseudopterosins (Ata *et al*., 2004; Correa *et al*., 2011), less information is known regarding their activity against ecologically relevant bacteria (Correa *et al*., 2012). Hence, this investigation also tested the hypothesis that ecologically relevant bacteria isolated from *P. elisabethae* would also be inhibited by pseudopterosin G (PsG).This analog was selected as it was previously amongst the most active pseudopterosin investigated against *Staphylococcus aureus* and *Enterococcus faecalis* (Correa *et al*., 2011).

Consistent with the earlier description and to avoid confusion between bacteria isolated by cultivation and gene sequences generated from bTEFAP, the term "isolates" herein refers to bacteria obtained by cultivation on solid media. The term 'library' is used to represent the collection of sequences identified from bTEFAP and 'phylotypes' or 'operational taxonomic

units (OTUs) to represent a group of PCR amplicon sequences considered to be essentially identical at the species ($OTU_{0.03}$; 97% sequence identity) or class level ($OTU_{0.10}$; 90% sequence similarity).

MATERIALS AND METHODS

Coral collection and initial processing: San Salvador, Bahamas (BS), 2008

Two colonies of *P. elisabethae* were identified by morphological characteristics and collected by R. Kerr by SCUBA from San Salvador Island at Runway 10, Bahamas ((BS), geographic coordinates: 24 03.898 N; 74 32.635 W), in August 2008. Precautions were taken to avoid contaminating coral specimens by the use of sterilized gloves, scissors, and polypropylene tubes as described previously (Chapter II). At the surface, the coral was cut into approximately 1 cm lengths and was washed three times with sterile filter seawater (SFSW, sterilized by autoclaving) to remove loosely associated microbes. Coral fragments were cut into thin sections using a sterile scalpel and ground into a homogenate using a microcentrifuge tube and pestle.

Plating methods: San Salvador, BS, 2008

For isolates denoted by 'RKBH', ground coral fragments from one coral colony were suspended in nine mL of SFSW and diluted to 10^{-3} and 10^{-5} using SFSW. From the 10^{-5} dilution, 100 µL was plated in triplicate on individual Bacto Marine Agar 2216 (MA, Difco BD Biosciences, Franklin Lakes, NJ) plates, while 100 µL of the 10^{-3} dilution was pipetted on three plates of each of the media listed in Table 3.1. For V8 and R2A media, a sterile 0.45 µm nitrocellulose mixed ester membrane (GE: E04WP09025) was aseptically placed on the plates prior to inoculation to selectively isolate Actinobacteria (Hirsch and Christensen, 1983). Plates were wrapped in parafilm and shipped from San Salvador (SS) to the University of Prince Edward Island (UPEI)

between August 16 – 25, 2009. Once plates arrived at UPEI, emerging colonies were initially subcultured to the same medium as the isolation plates while further subculturing was performed on MA. "RKBH" cultures were plated on media indicated by an asterisk in Table 3.1. For 'SS' isolates, coral fragments from the second colony were finely chopped using a sterile razor and were homogenized using a microcentrifuge tube and pestle, adding 75% ASW (prepared at 35 g/L) to aid homogenization. Homogenate was placed in a sterile Nalgene cryovial, and sterile glycerol was added to coral homogenates to a concentration of 25% (v/v). Samples were mixed well and flash frozen in a liquid nitrogen dry shipper for transport back to UPEI, storing samples at -80°C upon arrival. Samples were thawed and were diluted from up to 10^{-3} using 75% ASW (at 35 g/L) and 25% MilliQ® water. For all three dilutions, 100 µL was plated in triplicate on media shown in Table 3.1 (n = 120 plates), yet were not plated on V8 agar or No Nal R2A (indicated by a '+' in Table 3.1).

Coral collection and initial processing: Bimini, BS, 2009

Three specimens of visibly healthy *P. elisabethae* colonies (replicates A, B, and C) were collected by SCUBA by R. Kerr at a collection depth of 50 – 60 ft from two reefs surrounding Bimini, the Bahamas (Victory Reef = Site 1 – 25 29.13′ N 79 16.41′ W; Tuna Alley = Site 2 –25 31.38′ N 79 16.41′ W) in February 2009. Corals were identified by morphological characteristics and were collected 33 ft apart to ensure clonal propagates were not re-sampled. Multiple branches of the same individual corals were collected by inverting a sterile 50 mL polypropylene tube underwater and cutting specimens with pre-sterilized scissors, being careful to avoid touching the coral section to be analyzed. At the surface, corals were aseptically cut into ~0.5 – 1 cm lengths using sterilized scalpels and were transferred to new sterile 50 mL tubes, filling the tubes halfway with coral fragments. To remove loosely associated microorganisms, coral

Table 3.1: Media ingredients for the isolation of bacteria from *P. elisabethae* from San Salvador, BS (2008). Media was sterilized by autoclaving and antimicrobials were filter-sterilized and added post-autoclaving. Purification agar for all agars except MA (prepared in MilliQ® water) was prepared using 75% ASW (Instant Ocean, 18 g/L).

Isolation Media	Description/Reference	Composition/supplier
Marine agar (MA)*	Medium for the enumeration of salt-requiring or salt tolerant bacteria	BD Difco™, 0.66 µg/mL cyclosporine A and 50 µg/mL Nystatin, MilliQ® water to 1 L.
Dilute MA	Diluted 100 fold as previous studies have demonstrated that the number of morphologically distinct bacteria increased by 20% when dilute MA was selected over full strength media (Bruck *et al*., 2007)	BD Difco™ premix, diluted 1/100; 10 g/L agar, 50% ASW to 1 L; 50 µg/mL cyclohexamide.
R2A*	Used for the isolation of slow-growing, chlorine-tolerant bacteria. Nalidixic acid used at a low concentration to act as a bacteriostatic agent (Deitz *et al*., 1966)	BD Difco™, 10 µg/mL nalidixic acid, 50 µg/mL cyclohexamide, 50 µg/mL nystatin; 1 L ASW
No Nal R2A*+	As above, no nalidixic acid added	BD Difco™, 50 µg/mL cyclohexamide, 50 µg/mL nystatin; 1 L ASW
dR2A	Solidified with an alternative gelling agent as a means to increase the number of colony formations (Tamaki *et al*., 2009).	As above for R2A, BD Difco™, diluted 1/100; 15 g/L gellan gum; 1 L ASW.
V8 Agar*+	A medium used to stimulate the production of fungal sexual spores (Kent *et al*., 2008; Campbell soup Co.)	20 g/L agar, 4 g/L $CaCO_3$, 20 mL V8 juice, ASW to 1L, 10 µg/mL nalidixic acid, 50 µg/mL cyclohexamide, 50 µg/mL nystatin, to 1 L ASW, pH 7.3
ISP-4	International *Streptomyces* project medium, a selective isolation media	BD Difco™, ASW to 1 L
M3	M3 was selected owing to its ability to increase the recovery of Actinobacteria from three sponge species based on colony morphotypes	50 µg/mL cyclohexamide; 10 g/L agar; 1 L ASW
C media	Selected owing to its ability to produce the highest cultivable diversity from bacterial communities associated with a South China Sea sponge (Li *et al*., 2007)	50 µg/mL nalidixic acid
dNA	A high nutrient non-selective medium diluted to enhance the cultivability of bacterial isolates (Janssen *et al*., 2002)	BD Difco™ premix diluted 1/1000; 10 g/L agar

An asterisk (*) represents culture media for RKBH isolates; '+' refers to isolation media not used for the isolation of 'SS' bacteria.

fragments were shaken vigorously three times with SFSW. Coral pieces were homogenized using a sterilized homogenizer, where the homogenizer was sterilized after each sample by 10% bleach, followed by 70% EtOH, and rinsed using SFSW. Coral homogenates were stored at 4°C for 3 – 6 hours prior to plating.

Isolation media and plating methods: Bimini, BS, 2009

As metagenomic analysis of a stony coral previously revealed associated bacteria were generally heterotrophic (Wegley *et al*., 2007), media which generally targeted such bacteria were selected. The ultimate goal of the research project was to maximize the culturable diversity to provide a platform for analyzing the biomedical potential of *P. elisabethae*-associated bacteria by analysis of fermentation extracts. Consequently, the bacterial diversity was maximized by incorporating a variety of isolation media with distinct nutrients (Table 3.2). Bacteria were plated on isolation media following application of the PFT (described below). BG-11 plates were stacked layer-deep on the window side and stored at room temperature (~20°C) to expose photosynthetic Cyanobacteria to a source of light. Plates were individually wrapped in Parafilm and were transported back to UPEI and incubated at room temperature (~23°C) for up to four months to enable the growth of slow growing colonies (Ben-Dov *et al*., 2009) and checked periodically for the emergence of new colonies. Isolates were subcultured two to three times to ensure purity, and re-streaking of isolates was performed on MA. To archive isolates, cultures were grown in sterilized Marine Broth (MB, BD Difco™) for 24 – 48 hours, and 25% glycerol (v/v) was added to 500 µL of cultures in MB which were archived at -80°C.

Table 3.2: Media ingredients for the isolation of bacteria from *P. elisabethae* from Bimini, BS (2009). Media was sterilized by autoclaving and antimicrobial agents were filter-sterilized and added post-autoclaving. Purification agar for all agars except MA (prepared in MilliQ® water) was prepared using half strength Instant Ocean (ASW, 18 g/L).

Isolation Media	Rationale	Supplier/Composition
MA	Refer to Table 3.1	BD Difco™, Franklin Lakes, NJ, USA; 50 µg/mL cyclohexamide
Dilute R2A	Refer to Table 3.1	BD Difco™ Diluted 1/100, 15 g/L gellan gum, 10 mM filter-sterilized (0.22 µm) $MgSO_4 \cdot 7H_2O$, 50 µg/mL cyclohexamide; pH 7.2.
C media	Refer to Table 3.1	Refer to table 3.1
M3	Refer to Table 3.1	10 g/L agar, 50 µg/mL nalidixic acid
***Pseudomonas* specific media (Ps)**	A selective medium for the enumeration of *Pseudomonas* sp., an isolate potentially responsible for pseudopterosin production in *P. elisabethae* (Bunyajetpong, 2011).	10 g K_2SO_4, 1.4 g $MgCl_2 \cdot 6H_20$, 20 g peptone, ASW (to 1 L), 50 µg/mL cyclohexamide, 50 µg/mL nystatin, 25 µg/mL Irgasan, 10 µg/mL nalidixic acid, 13.6 g agar, pH 7.0
PE (Invertebrate specific media)	Used with the rationale that uncultivable microbes may grow in pure culture if provided with cues from the natural environment (Webster and Hill, 2001)	Top layer: 10 g of finely ground *P. elisabethae*, 5 g agar, 750 mL 35% ASW, 250 mL MilliQ® water. Bottom layer: 750 mL ASW, 250 mL MilliQ® water, 15 g/L agar, and 50 µg/mL cyclohexamide.
Artificial seawater (ASW)	To enable the growth of potentially oligotrophic bacteria	750 mL ASW, 250 mL MilliQ® water, 15 g/L agar, and 50 µg/mL cyclohexamide, pH 7.6.
BG-11	A selective medium targeting Cyanobacteria, which are excellent producers of bioactive metabolites (Williams, 2009)	1.5 g $NaNO_3$, 40 mg K_2HPO_4, $MgSO_4 \cdot 7\ H_20$, 36 mg $CaCl_2 \cdot 2\ H_20$, 6 mg Citric acid, 6 mg Ferric ammonium citrate, 1 mg EDTA (disodium salt), 20 mg Na_2CO_3, 1mL trace metal mix, 100 mL vitamin mix, 1 L ASW, 10 g agar, 50 µg/mL cyclohexamide. Trace metals mix: 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_20$, 0.222 g $ZnSO_4 \cdot 7H_20$, 0.39 g $NaMoO_4 \cdot 2\ H_20$, 79 mg $CuSO_4 \cdot 5H_20$, 49.4 mg $Co(NO_3)_2 \cdot 6H_20$, sterile MilliQ® water (to 1L); Vitamin mix: 0.1 g Thiamine HCl, 0.025 mg Biotin, 0.015 mg Cyanocobalamin prepared in 1 L MilliQ® water, filter sterilized, added post-autoclave. pH 7.1

Isolation strategy: Particle filtration technique (PFT, Bimini, BS, 2009)

Briefly, the PFT involves subjecting coral homogenates to vacuum filtration through mesh of different sizes and plating filtrates from selected screens on agar. The filtration apparatus (minisieve set, Scienceware®) was assembled with mesh screens placed in descending order (500 µm, 213 µm, 104 µm, and 51 µm; Figure 3.1). Theoretically, large sized tissue particles (including coral fragments) will be trapped on the uppermost screens while small tissue particles and free bacterial cells will flow through each screen culminating in the particle filtrate. Homogenized coral samples were poured onto the uppermost brass pre-screen, and the apparatus was covered with a lid. Vacuum was applied using a vacuum pump to pull particles through the screens, where the particle filtrate collected from the filter flask was poured into a 50 mL tube and placed on ice. The particles retained on the 104 µm and 51 µm screens were removed using sterile forceps and were placed in sterile 50 mL centrifuge tubes containing 30 mL sterile filter seawater (SFSW), which was prepared by filtering seawater collected on location using a 0.22 µm Nalgene MF575 water filtration device (VWR International, Mississauga, ON). Samples were vortexed to remove particles from screens, and particles were carefully scraped from the screens using sterile forceps.

To increase the bacterial diversity of *P. elisabethae*, the dilution-to-extinction protocol was used (Button *et al*., 1993). This procedure has distinct advantages by enabling the isolation of oligotrophic bacteria, which otherwise become overshadowed by quickly-growing isolates (Button *et al*., 1993). Particle suspensions were poured into a sterile Petri dish containing a sterile stir bar, situated on a stir plate to keep particles in suspension. Dilutions from the different mesh screens were vortexed in an appropriate amount of SFSW prior to plating (Appendix Table B1). Either 10 or 20 µl (Table B1) of the dilutions of each particle size was pipetted per well by a

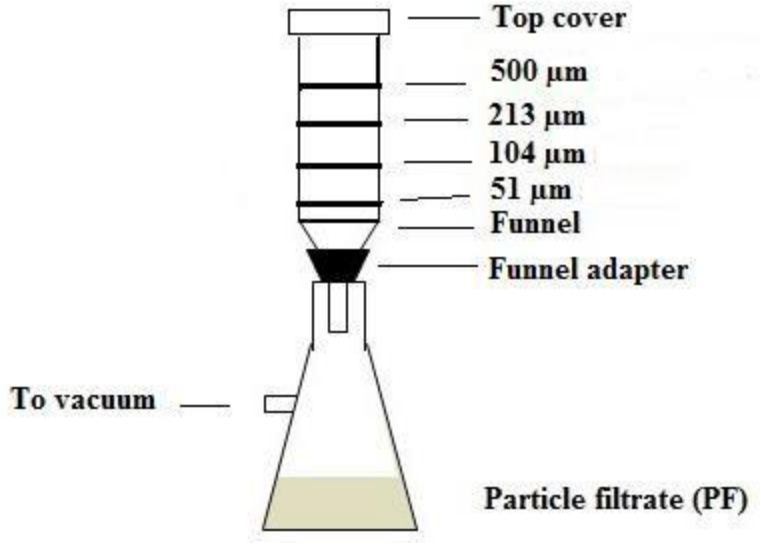


Figure 3.1: The particle filtration device employed in the isolation of *P. elisabethae*-associated bacteria.

multichannel pipettor onto one plate of each isolation media (contained in 48-well polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ). A control of 20 µL per well of SFSW was pipetted into well A1 of each plate.

Designation of isolates

Isolates obtained from San Salvador in 2008 were denoted by the prefix 'SS' if cultured by V. Robertson, or by 'RKBH' if purified by B. Haltli. Isolates obtained from fresh coral homogenate (2009 collections) and cultured by V. Robertson are labeled as "RKVR" isolates or are represented by a single alphabetical letter followed by a number. 'SS' isolates were prepared from frozen coral fragments, while 'RKBH' and isolates designated by 'RKVR' or a single alphabetical letter were prepared from fresh coral homogenate.

Bacterial genomic DNA extraction protocol: San Salvador, BS, 2008

Genomic DNA (gDNA) from 2008-collected coral isolates were extracted using a similar approach. However, 2009-collected coral isolates were first dereplicated by MALDI-TOF MS prior to DNA extraction; the DNA extraction procedure is as described for San Salvador isolates. Isolates were grown on MA overnight, and single colonies were picked with a toothpick and dispersed in 25 µL of DMSO as a rapid method of lysing bacterial cells. DMSO cell suspensions were used as a template in PCR reactions. However, if PCR reactions were unsuccessful using this method, DNA was isolated from bacterial cultures using a generic phenol:chloroform extraction protocol. Briefly, cells grown on MA were cultured in 5 mL of MB broth at 30°C until confluent growth was observed. A portion (500 µL) of each culture was pipetted into a 1.5 mL microcentrifuge tube. Cells were pelleted at 10,000 rpm using a desktop centrifuge (Sorvall Biofuge pico Thermo Scientific) for 5 minutes, the supernatant was removed, and cells were

resuspended using 1 mL of sterile MilliQ® water. Water was removed following cell pelleting as above, and cells were suspended in 300 µL 50/20 TE (50 mM Tris, 20 mM EDTA, pH 8.0). Cells were incubated with 5 mg/mL lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 1 µL RNase ONE™ (Promega, Madison, WI) for 30 minutes at 37°C. Following incubation, 50 µL of 10% sodium dodecyl sulfate (SDS) and 85 µL of 5 M NaCl was added and the sample was mixed well. A 500 µL volume of phenol/chloroform:isoamyl alcohol (25:24:1) was added to each tube and vortexed for 30 seconds. The aqueous layer was transferred to a new tube, where DNA was precipitated using 500 µL of isopropanol. Samples were mixed by inversion and incubated at room temperature for five minutes. DNA was pelleted at 10,000 rpm using a desktop centrifuge (Sorvall Biofuge pico) and isopropanol was removed. DNA pellets were washed with 500 µL of ice-cold EtOH to remove excess salt. EtOH was removed following centrifugation as above and DNA was dried in a laminar flow cabinet for 10 minutes at room temperature. DNA was re-suspended in Tris-HCl (pH 8.0) and stored at -20°C.

PCR amplification of 16S rDNA sequences from isolate gDNA: San Salvador, BS, 2008

Bacterial 16S rRNA gene sequences were amplified from 2008-collected coral isolates genomic DNA using the universal primers pA and pH (Edwards *et al*., 1989). PCR reactions contained 1X EconoTaq® Plus Green Master Mix (Lucigen, Middleton, WI, USA) supplemented with 2.5 - 5% molecular biology grade DMSO (Sigma, Oakville, ON), 1 µM of each primer, 20 ng of template DNA, and sterile MilliQ® water to 25 µL. Thermal cycling parameters were as follows: 1 cycle at 95ºC for 3 min; 34 cycles of 95ºC for 45 sec, 54ºC for 1 min, and 72ºC for 1.5 min, followed by a final extension cycle at 72ºC for 5 min. PCR amplification was assessed by 1% agarose gel electrophoresis (AGE, 1X TAE, 1% agarose w/v). PCR products were purified following band

excision at ~1,500 bp if non-specific banding was detected by 1% AGE. Bands were purified from the agarose using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

RFLP dereplication of isolates obtained from *P. elisabethae*: San Salvador, BS, 2008

PCR amplicons of 16S rDNA sequences from 'RKBH' and 'SS' isolates were digested with *Hha*I and *Hae*III separately according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). RKBH isolates were dereplicated by B. Haltli. Restriction fragments were analyzed by AGE, where gels were prepared with 1.5% agarose (w/v). Gels were electrophoresed at 110V for 1hr30 min. Ten microliters of each digest was loaded with 2 µL of 6X loading dye (Fermentas) and loaded into the gel. A 2-log ladder (NEB) was used as a molecular weight standard. Isolates exhibiting the same restriction fragment patterns with both enzymes were considered to be identical.

Whole-cell MALDI-TOF MS dereplication of coral-associated bacteria: Bimini, BS, 2009

Prior to gDNA extraction and PCR amplification, isolates from 2009-collected corals were first dereplicated to eliminate isolate redundancy and select for novelty from coral-associated bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Although morphological characteristics are a poor method of bacterial dereplication (Dieckmann *et al*., 2005), the initial dereplication relied upon differences in colony morphology and pigmentation as a means to reduce the number of morphologically similar isolates. Isolates were prepared by scraping a small amount of archived bacterial cells with a sterile toothpick from glycerol stocks onto a 96-well plate filled with 250 – 300 µL of MA. Since the growth stage of bacteria can influence the MALDI-TOF MS spectral output (Vargha *et al*., 2006),

experimental conditions were standardized by growing bacteria at 48 ± 2 hours at room temperature (~22°C). The matrix, consisting of 500 µL acetonitrile, 475 µL sterile MilliQ® water, 25 µL trifluoroacetic acid (TFA), and α-cyano-4-hydroxycinnamic acid (CHCA) to saturation, was prepared fresh in a sterile 1.5 mL microcentrifuge tube. A TFA concentration of 2.5% (v/v) was selected as previous studies demonstrated this improves ionization and spectrum quality for whole cell MALDI of both Gram-positive and Gram-negative bacteria (Vargha *et al*., 2006). The matrix was vortexed for 30 seconds, centrifuged for 2 minutes at 10,000 rpm, and stored at 4°C until needed. Using a sterile wooden applicator, a thin smear of bacteria was stamped from isolates growing on marine agar to a Brüker stainless steel MSP 96 target plate, taking care to avoid oversaturation of the target spot. MALDI matrix (1.2 µL) was added to the top of the bacterial smear, and isolates were air dried at room temperature. The air dry method has previously been demonstrated to generate the best quality MALDI spectra in terms of number of detected signals and signal-to-noise ratio (Vaidyanathan *et al*., 2002). Duplicates of selected colonies were analyzed A) on different days and B) the same plate to ensure quality monitoring. *E. coli* was added as a control to each target plate. The use of MALDI-TOF MS was kindly provided by Dr. J. Lewis and technical assistance was provided by B. Despres.

Bacterial protein fingerprints were generated from isolates using a Microflex LT MALDI-TOF MS (Brüker Daltonics) equipped with a 50-Hz N_2 laser. MS analyses were performed in linear, positive ionization mode with a *m/z* charge selection in the range of 2,000 – 20,000 and an acceleration voltage of 20 kV. FlexControl software (Brüker Daltonics) was used to generate MALDI-TOF MS peak profiles. Optimal instrument settings were empirically determined. Briefly, the laser intensity was adjusted to a minimum of 50%, and the total number of laser shots per spot was adjusted to a minimum of 200. A minimum "summation" intensity of

500 – 1,000 was ensured for each sample, with the laser intensity adjusted to 60% if necessary. Cluster analysis from MALDI-generated spectra was constructed selecting Unweighted Paired Group Method with Arithmetic mean (UPGMA) using Biotyper software (version 2.0, Brüker Daltonics), since clustering by UPGMA has been previously demonstrated to suitably group similar isolates at the species-to-strain level (Ghyselinck *et al*., 2011). Dereplication parameters for environmental isolates have not been defined by Biotyper bacterial identification software (Brüker Daltonics). Furthermore, as the distance level for defining "unique" (species-level) bacteria is unknown, sequencing effort was prioritized by sequencing representative isolates from clusters in the dendrogram with a similarity cutoff of ≥ 20. The flow-through procedure for MALDI-TOF MS dereplication is summarized in Figure 3.2.

Genomic DNA extraction and PCR amplification: Bimini, BS, 2009 isolates

Genomic DNA extraction and subsequent PCR amplification for MALDI-TOF MS de-replicated isolates was performed as described previously for 2008-collected San Salvador isolates.

Sequencing of 16S rDNA amplicons: Bimini; San Salvador, BS

Sequencing of all isolates was performed by Genome Québec (Montréal, QC) or the Princess Margaret Hospital (Toronto, ON) using the 16SR530 primer (5'-GTA TTA CCG CGG CTG CTG-3'), which provides a comparative region on the 16S rRNA gene to that sequenced from bTEFAP data generated from *P. elisabethae* associated bacteria (Chapter II). Only high quality sequences with less than five ambiguous nucleotides ('N') and sequences ≥250 bp were kept in the analysis. Chimeras were checked using Bellerophon using default program parameters (Huber *et al*., 2004). Full length sequences were obtained for isolates with less than 96% identity

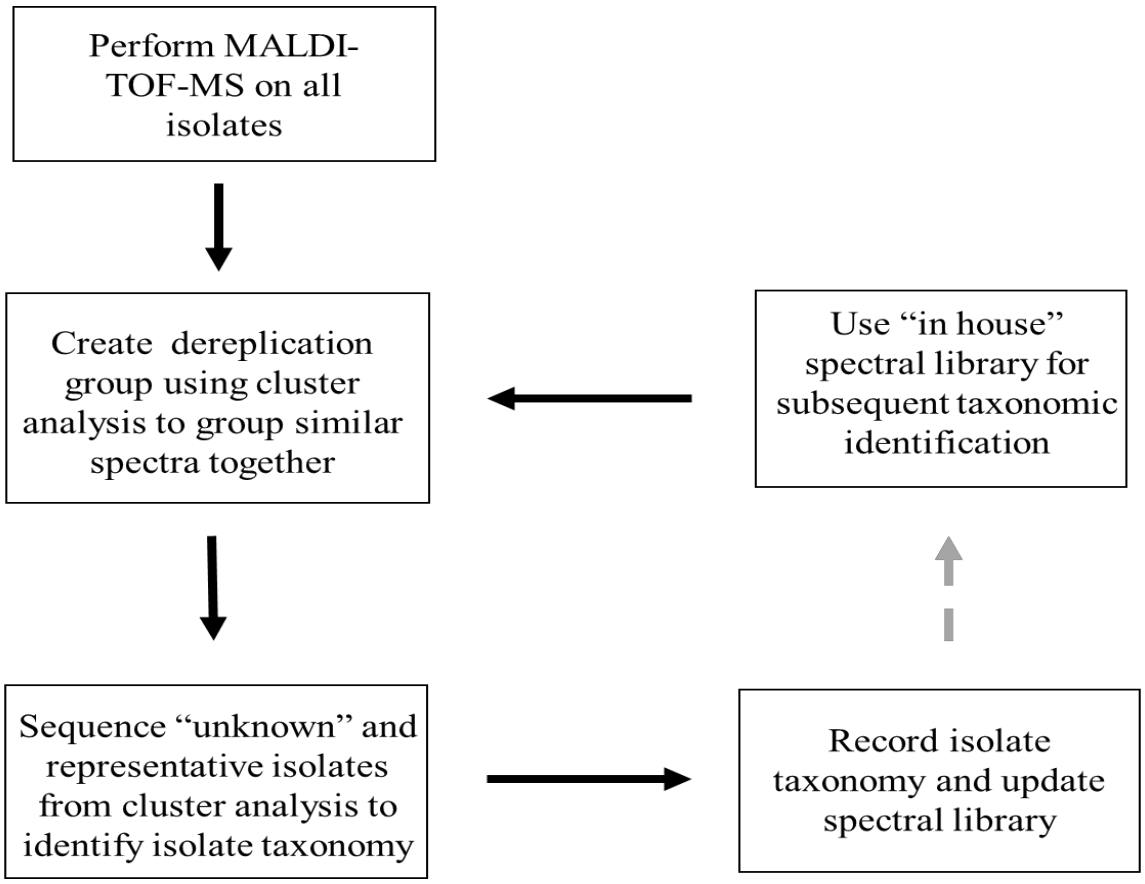


Figure 3.2: MALDI-TOF MS library dereplication procedure.

to published sequences. Isolates were submitted to GenBank under the accession numbers JX317679 – JX317743 and JX407110 – JX407263 (Table B2).

Taxonomic identification of 16S rDNA sequences: Bimini and San Salvador, BS

All bacterial isolates from *P. elisabethae* were identified by comparison of partial 16S rRNA gene sequences to published sequences in GenBank, using either the Reference RNA sequences (refseq_rna) or nucleotide collection (nr/nt) database. Isolates from both collection years (2008 and 2009) and from both preparation methods (plating fresh versus frozen coral homogenate) were combined together for the analysis.

Phylogenetic analysis of *P. elisabethae*-associated bacteria

In order to articulate the level of bacterial diversity associated with *P. elisabethae*-associated isolates, a phylogenetic tree of 16S rDNA sequences was constructed. 16S rRNA gene sequence amplicons were aligned using ClustalW in MEGA 5.05 (Tamura *et al*., 2011). Aligned sequences were assessed for evolutionary similarity using a neighbour-joining phylogenetic tree in MEGA 5.05. Bootstrapping (n = 1,000) was used to assess overall tree accuracy and aligned nucleotide sequence gaps were treated by complete deletion. Trees were rooted using different 16S rDNA sequences from representative organisms. Overall tree topologies were validated by reconstructing phylogenies using multiple evolutionary distance methods including neighbour-joining (gaps treated by complete deletion), maximum likelihood, minimum evolution, and UPGMA.

Comparison of 16S rDNA sequences from the culturable library to the culture-independent library of *P. elisabethae*

Partial 16S rDNA sequences of cultured isolates were compared to 13 PCR-generated bTEFAP sequence libraries (1A – 1D, 2A – 2B, and 3B – 3E, refer to Table 2.1 (Chapter II) for descriptions) by clustering sequences to the species level (97% sequence identity) in the Ribosomal Database Project II (RDP-II, Cole *et al*., 2009). Cultures were taxonomically assigned using a 50% confidence threshold, which is suitable for accurate taxonomic assignment of short sequences and classifies a higher number of sequences than more stringent confidence thresholds (Claesson *et al*., 2009). When comparison to a single bTEFAP sequence library was necessary, sample 3E was selected as a representative since this individual was collected in the same year/site (site 2 in culture studies) as many of the cultured isolates. The combined cluster file was assessed for overlapping $OTU_{0.03}$s between cultures to pyrosequences in Excel (data not shown). Any sequence present in both culture and culture-independent sequencing libraries were considered to be 'overlapping' $OTU_{0.03}$s. To determine the number of unique $OTU_{0.03}$s, sample 3E was de-replicated to 97% sequence similarity using Fast-Group-II (FG-II; Seguritan and Rohwer, 2001).

Sensitivity and inhibition of coral-associated bacterial isolates to pseudopterosin G

Agar plates were prepared by adding 0, 5, or 50 mg/L of purified PsG prepared in 50% EtOH to MA aseptically following autoclaving. Previously growing cultures were transferred onto pseudopterosin-containing agar, and growth rates were recorded. Isolates were considered as 'sensitive' or 'restricted' to PsG if their growth was compromised, but not completely inhibited,

while isolates unable to grow in the presence of PsG were termed ‘inhibited. Pseudopterosin sensitivity tests were performed by N. Duncan.

Salinity requirements of *P. elisabethae*-associated bacteria

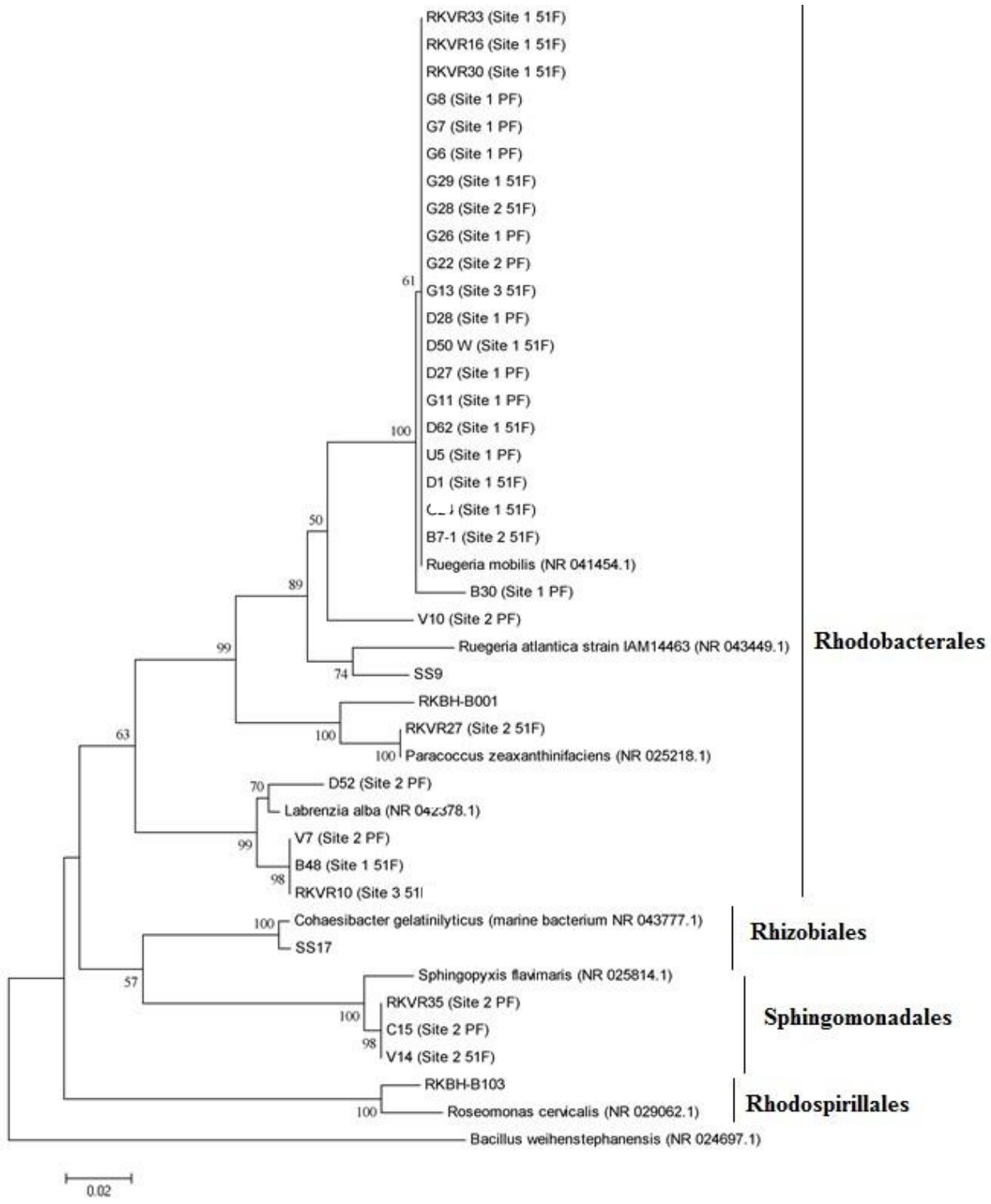
Selected isolates were also assessed for their salinity requirements. To verify purity and viability and to determine the salt requirement of each isolate, cultures were streaked initially on MA, which contains 19.45 g/L sodium chloride to simulate natural sea water concentrations. Plates were incubated at 30°C, and cultures were transferred using a sterile flat-tip toothpick to Salt Free Marine Agar (SFMA, 5 g/L peptone, 1 g/L yeast extract, 0.1 g/L ferric citrate, 15 g/L agar). Isolates were also transferred to fresh MA plates to ensure cultures remained viable. Isolates unable to grow on SFMA were considered marine (Jensen and Fenical, 1994). Salinity tests were performed by N. Duncan.

RESULTS AND DISCUSSION

The culturable bacterial community of *P. elisabethae*

The aim of this study was to explore the cultivable bacterial diversity from the octocoral *P. elisabethae*. To accomplish this goal, bacteria were isolated from *P. elisabethae* from two collection years (2008 and 2009), three collection sites (San Salvador (2008), site 1 and site 2, Bimini (2009), refer to Chapter II, Figure 2.1), and from two homogenate types (fresh and frozen coral homogenate). Since one of the goals in this research was to maximize the diversity of cultures from homogenized *P. elisabethae* tissue, all isolates obtained from each collection year and site were included in the analysis. Although one might argue that combining bacterial isolates from different collection years may provide erroneous measures of diversity, previous studies examining samples collected at the same time and from the same colony in stony corals has demonstrated bacterial communities differ dramatically between cultures and molecular techniques regardless (Lampert *et al*., 2008; Rohwer *et al*., 2001), therefore, it is rationalized that combining isolates will not significantly affect measures of diversity and could increase the overlap of bacteria obtained from each methodology. Innovative culturing techniques (diverse isolation media and the PFT) were also applied to 2009 collected samples to determine if the diversity of bacterial isolates obtained from *P. elisabethae* could be increased. From the entire cultivable bacterial community, phylogenetic investigation of MALDI-TOF MS and RFLP-dereplicated bacterial isolates (n = 222) revealed the *P. elisabethae* community represented four bacterial phyla and five classes (Figure 3.3). Specifically, the most abundant bacteria at the class level were Gammaproteobacteria, Alphaproteobacteria, Bacilli, Actinobacteria, and

A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G8 (Site 1 PF)
G7 (Site 1 PF)
G6 (Site 1 PF)
G29 (Site 1 51F)
G28 (Site 2 51F)
G26 (Site 1 PF)
G22 (Site 2 PF)
G13 (Site 3 51F)
D28 (Site 1 PF)
D50 W (Site 1 51F)
D27 (Site 1 PF)
G11 (Site 1 PF)
D62 (Site 1 51F)
U5 (Site 1 PF)
D1 (Site 1 51F)
B7-1 (Site 2 51F)
Ruegeria mobilis (NR 041454.1)
B30 (Site 1 PF)
V10 (Site 2 PF)
Ruegeria atlantica strain IAM14463 (NR 043449.1)
SS9
RKBH-B001
RKVR27 (Site 2 51F)
Paracoccus zeaxanthinifaciens (NR 025218.1)
D52 (Site 2 PF)
Labrenzia alba (NR 042378.1)
V7 (Site 2 PF)
B48 (Site 1 51F)
RKVR10 (Site 3 51
Cohaesibacter gelatinilyticus (marine bacterium NR 043777.1)
SS17
Sphingopyxis flavimaris (NR 025814.1)
RKVR35 (Site 2 PF)
C15 (Site 2 PF)
V14 (Site 2 51F)
RKBH-B103
Roseomonas cervicalis (NR 029062.1)
Bacillus weihenstephanensis (NR 024697.1)
61
100
50
89
99
74
63
100
100
70
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Rhodobacterales
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Rhodospirillales
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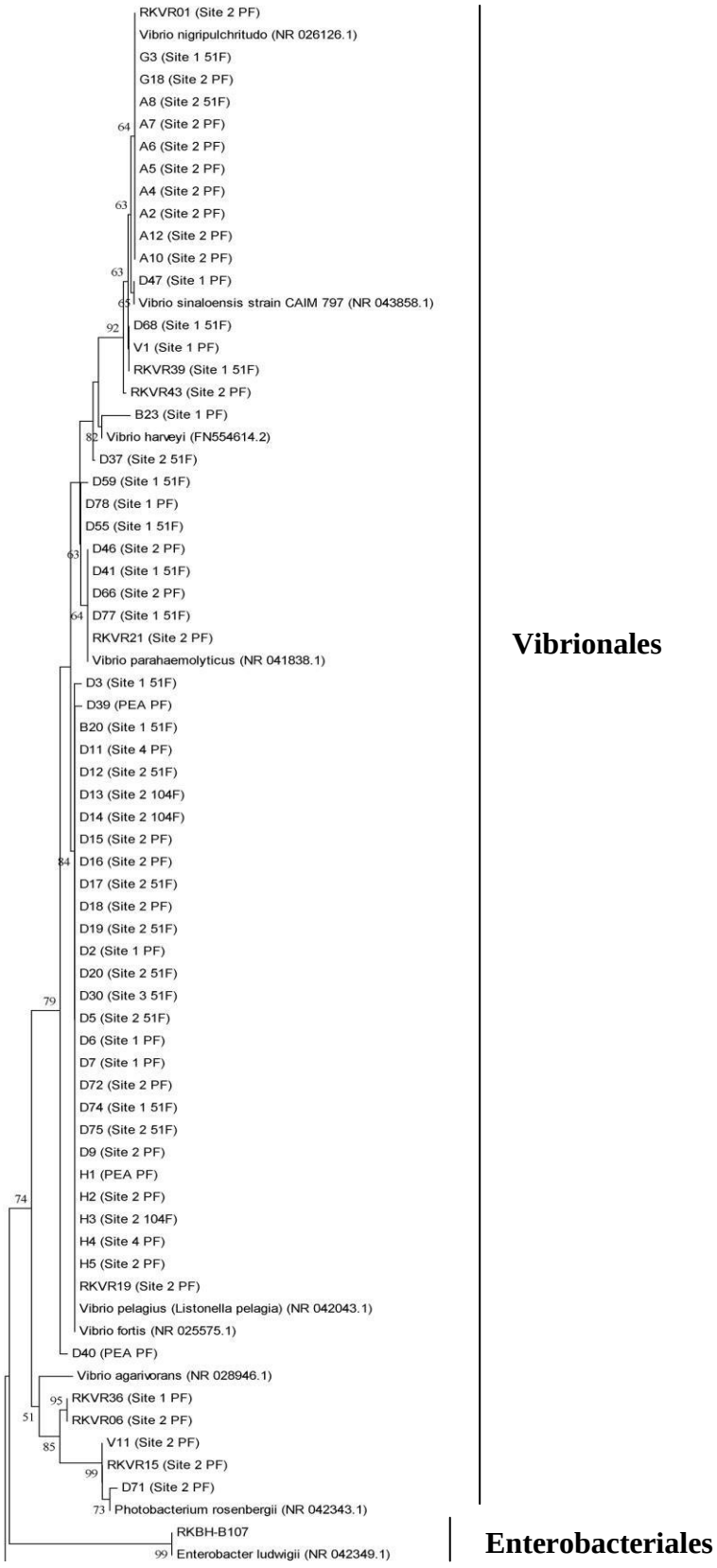
B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A8 (Site 2 51F)
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A7 (Site 2 PF)
A6 (Site 2 PF)
A5 (Site 2 PF)
A4 (Site 2 PF)
63
A2 (Site 2 PF)
A12 (Site 2 PF)
A10 (Site 2 PF)
63
D47 (Site 1 PF)
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Vibrio sinaloensis strain CAIM 797 (NR 043858.1)
92
D68 (Site 1 51F)
V1 (Site 1 PF)
RKVR39 (Site 1 51F)
RKVR43 (Site 2 PF)
B23 (Site 1 PF)
82
Vibrio harveyi (FN554614.2)
D37 (Site 2 51F)
D59 (Site 1 51F)
D78 (Site 1 PF)
D55 (Site 1 51F)
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D46 (Site 2 PF)
D41 (Site 1 51F)
D66 (Site 2 PF)
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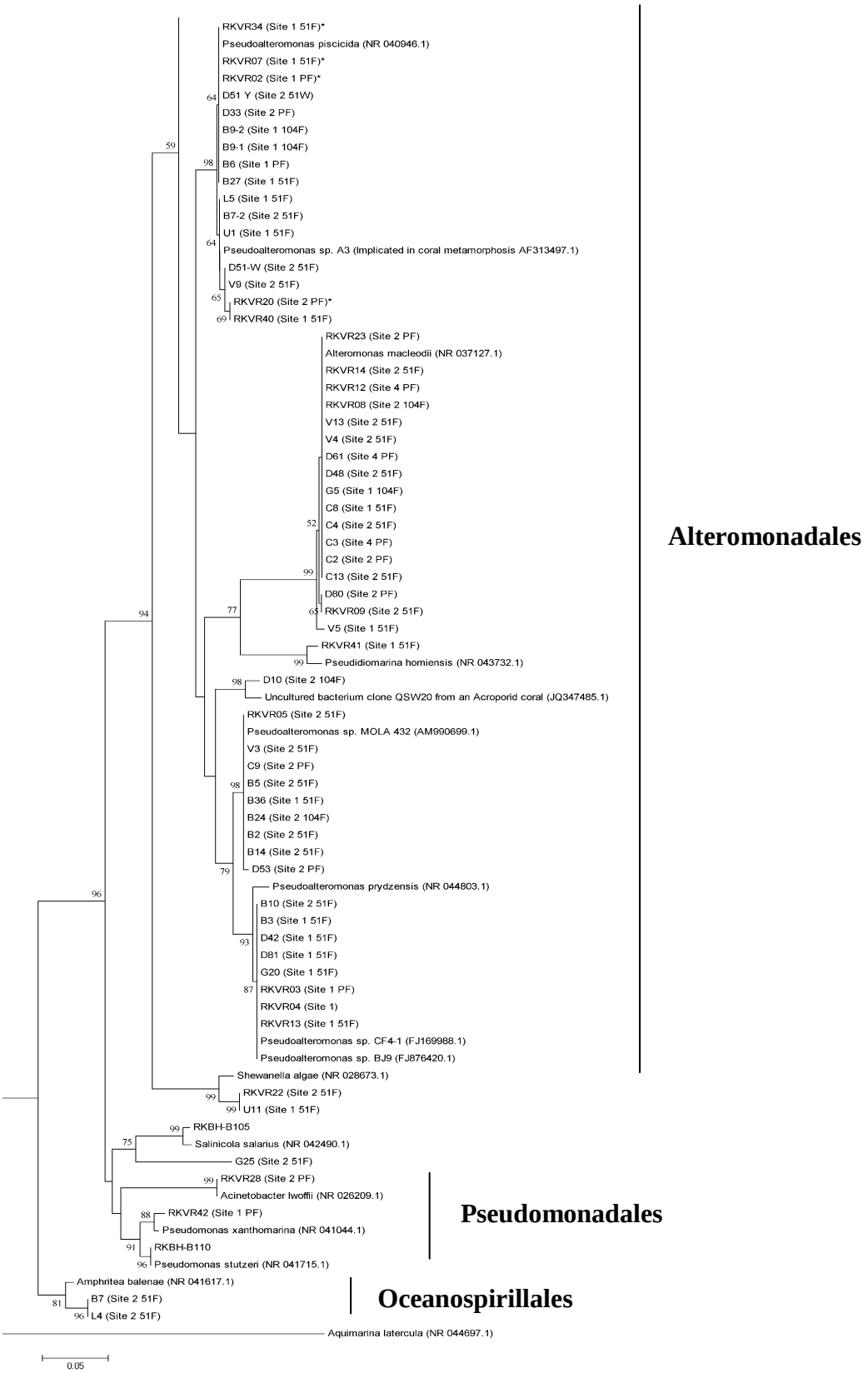
D77 (Site 1 51F)
RKVR21 (Site 2 PF)
Vibrio parahaemolyticus (NR 041838.1)
D3 (Site 1 51F)
D39 (PEA PF)
B20 (Site 1 51F)
D11 (Site 4 PF)
D12 (Site 2 51F)
D13 (Site 2 104F)
D14 (Site 2 104F)
D15 (Site 2 PF)
84
D16 (Site 2 PF)
D17 (Site 2 51F)
D18 (Site 2 PF)
D19 (Site 2 51F)
D2 (Site 1 PF)
D20 (Site 2 51F)
D30 (Site 3 51F)
79
D5 (Site 2 51F)
D6 (Site 1 PF)
D7 (Site 1 PF)
D72 (Site 2 PF)
D74 (Site 1 51F)
D75 (Site 2 51F)
D9 (Site 2 PF)
H1 (PEA PF)
74
H2 (Site 2 PF)
H3 (Site 2 104F)
H4 (Site 4 PF)
H5 (Site 2 PF)
RKVR19 (Site 2 PF)
Vibrio pelagius (Listonella pelagia) (NR 042043.1)
Vibrio fortis (NR 025575.1)
D40 (PEA PF)
Vibrio agarivorans (NR 028946.1)
95
RKVR36 (Site 1 PF)
51
RKVR06 (Site 2 PF)
85
V11 (Site 2 PF)
99
RKVR15 (Site 2 PF)
D71 (Site 2 PF)
73
Photobacterium rosenbergii (NR 042343.1)
RKBH-B107
99
Enterobacter ludwigii (NR 042349.1)
Vibrionales
Enterobacteriales

C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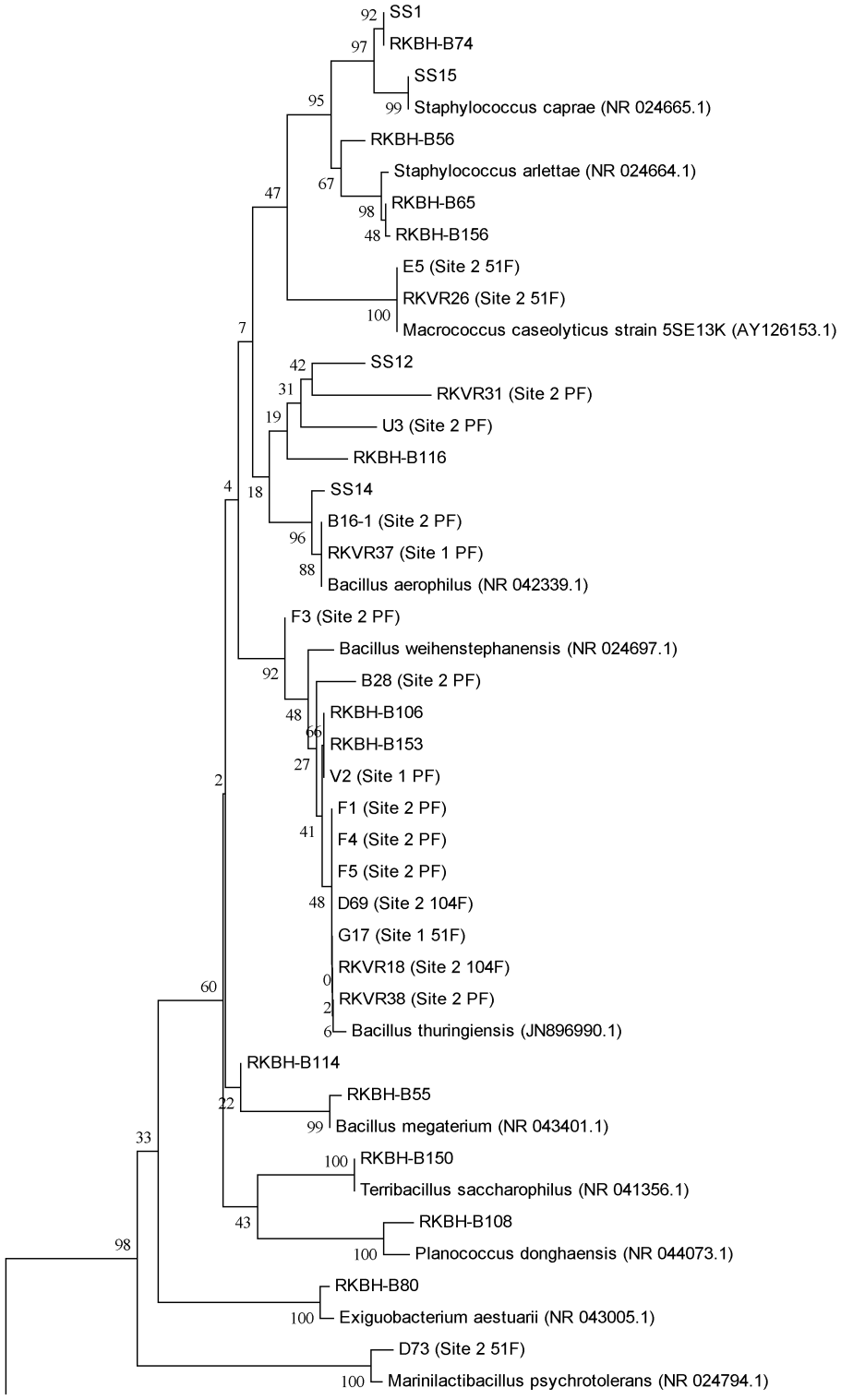
B9-2 (Site 1 104F)
B9-1 (Site 1 104F)
B6 (Site 1 PF)
B27 (Site 1 51F)
L5 (Site 1 51F)
B7-2 (Site 2 51F)
U1 (Site 1 51F)
Pseudoalteromonas sp. A3 (Implicated in coral metamorphosis AF313497.1)
D51-W (Site 2 51F)
V9 (Site 2 51F)
RKVR20 (Site 2 PF)*
RKVR40 (Site 1 51F)
RKVR23 (Site 2 PF)
Alteromonas macleodii (NR 037127.1)
RKVR14 (Site 2 51F)
RKVR12 (Site 4 PF)
RKVR08 (Site 2 104F)
V13 (Site 2 51F)
V4 (Site 2 51F)
D61 (Site 4 PF)
D48 (Site 2 51F)
G5 (Site 1 104F)
C8 (Site 1 51F)
C4 (Site 2 51F)
C3 (Site 4 PF)
C2 (Site 2 PF)
C13 (Site 2 51F)
D80 (Site 2 PF)
RKVR09 (Site 2 51F)
V5 (Site 1 51F)
RKVR41 (Site 1 51F)
Pseudidiomarina homiensis (NR 043732.1)
D10 (Site 2 104F)
Uncultured bacterium clone QSW20 from an Acroporid coral (JQ347485.1)
RKVR05 (Site 2 51F)
Pseudoalteromonas sp. MOLA 432 (AM990699.1)
V3 (Site 2 51F)
C9 (Site 2 PF)
B5 (Site 2 51F)
B36 (Site 1 51F)
B24 (Site 2 104F)
B2 (Site 2 51F)
B14 (Site 2 51F)
D53 (Site 2 PF)
Pseudoalteromonas prydzensis (NR 044803.1)
B10 (Site 2 51F)
B3 (Site 1 51F)
D42 (Site 1 51F)
D81 (Site 1 51F)
G20 (Site 1 51F)
RKVR03 (Site 1 PF)
RKVR04 (Site 1)
RKVR13 (Site 1 51F)
Pseudoalteromonas sp. CF4-1 (FJ169988.1)
Pseudoalteromonas sp. BJ9 (FJ876420.1)
Alteromonadales
Shewanella algae (NR 028673.1)
RKVR22 (Site 2 51F)
U11 (Site 1 51F)
RKBH-B105
Salinicola salarius (NR 042490.1)
G25 (Site 2 51F)
RKVR28 (Site 2 PF)
Acinetobacter lwoffii (NR 026209.1)
RKVR42 (Site 1 PF)
Pseudomonas xanthomarina (NR 041044.1)
RKBH-B110
Pseudomonas stutzeri (NR 041715.1)
Pseudomonadales
Amphritea balenae (NR 041617.1)
B7 (Site 2 51F)
L4 (Site 2 51F)
Oceanospirillales
Aquimarina latercula (NR 044697.1)
0.05

D


SS1
RKBH-B74
SS15
Staphylococcus caprae (NR 024665.1)
RKBH-B56
Staphylococcus arlettae (NR 024664.1)
RKBH-B65
RKBH-B156
E5 (Site 2 51F)
RKVR26 (Site 2 51F)
Macrococcus caseolyticus strain 5SE13K (AY126153.1)
SS12
RKVR31 (Site 2 PF)
U3 (Site 2 PF)
RKBH-B116
SS14
B16-1 (Site 2 PF)
RKVR37 (Site 1 PF)
Bacillus aerophilus (NR 042339.1)
F3 (Site 2 PF)
Bacillus weihenstephanensis (NR 024697.1)
B28 (Site 2 PF)
RKBH-B106
RKBH-B153
V2 (Site 1 PF)
F1 (Site 2 PF)
F4 (Site 2 PF)
F5 (Site 2 PF)
D69 (Site 2 104F)
G17 (Site 1 51F)
RKVR18 (Site 2 104F)
RKVR38 (Site 2 PF)
Bacillus thuringiensis (JN896990.1)
RKBH-B114
RKBH-B55
Bacillus megaterium (NR 043401.1)
RKBH-B150
Terribacillus saccharophilus (NR 041356.1)
RKBH-B108
Planococcus donghaensis (NR 044073.1)
RKBH-B80
Exiguobacterium aestuarii (NR 043005.1)
D73 (Site 2 51F)
Marinilactibacillus psychrotolerans (NR 024794.1)

E

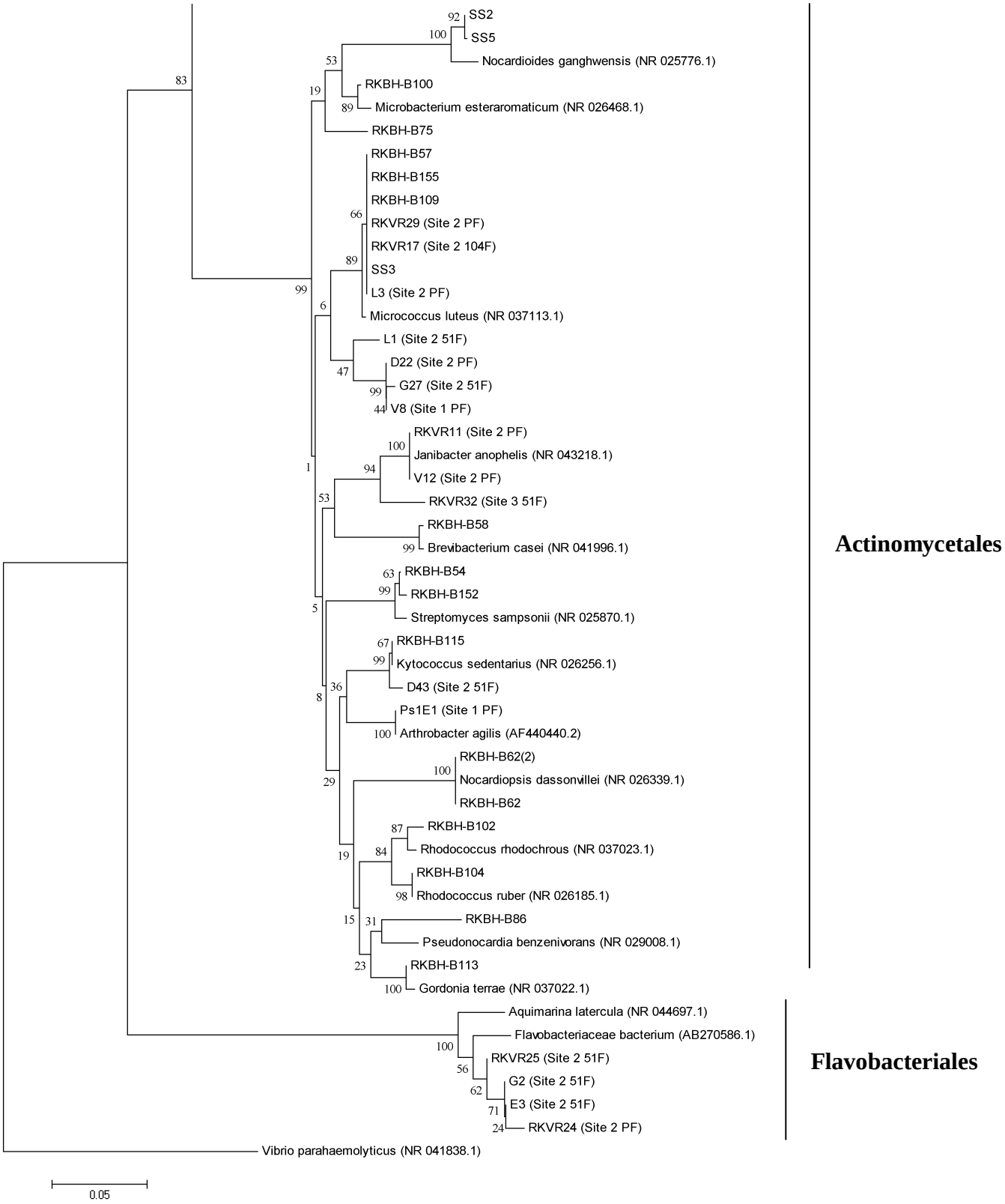

SS2
SS5
Nocardioides ganghwensis (NR 025776.1)
RKBH-B100
Microbacterium esteraromaticum (NR 026468.1)
RKBH-B75
RKBH-B57
RKBH-B155
RKBH-B109
RKVR29 (Site 2 PF)
RKVR17 (Site 2 104F)
SS3
L3 (Site 2 PF)
Micrococcus luteus (NR 037113.1)
L1 (Site 2 51F)
D22 (Site 2 PF)
G27 (Site 2 51F)
V8 (Site 1 PF)
RKVR11 (Site 2 PF)
Janibacter anophelis (NR 043218.1)
V12 (Site 2 PF)
RKVR32 (Site 3 51F)
RKBH-B58
Brevibacterium casei (NR 041996.1)
RKBH-B54
RKBH-B152
Streptomyces sampsonii (NR 025870.1)
RKBH-B115
Kytococcus sedentarius (NR 026256.1)
D43 (Site 2 51F)
Ps1E1 (Site 1 PF)
Arthrobacter agilis (AF440440.2)
RKBH-B62(2)
Nocardiopsis dassonvillei (NR 026339.1)
RKBH-B62
RKBH-B102
Rhodococcus rhodochrous (NR 037023.1)
RKBH-B104
Rhodococcus ruber (NR 026185.1)
RKBH-B86
Pseudonocardia benzenivorans (NR 029008.1)
RKBH-B113
Gordonia terrae (NR 037022.1)
Aquimarina latercula (NR 044697.1)
Flavobacteriaceae bacterium (AB270586.1)
RKVR25 (Site 2 51F)
G2 (Site 2 51F)
E3 (Site 2 51F)
RKVR24 (Site 2 PF)
Vibrio parahaemolyticus (NR 041838.1)
0.05
Actinomycetales
Flavobacteriales

Figure 3.3: Evolutionary relationship of *P. elisabethae*-associated bacterial cultures. Phylogeny was inferred using 16S rRNA gene sequences ≥400 bp by a neighbour-joining phylogenetic tree in MEGA 5.05 (Tamura *et al*., 2011) where evolutionary distances were calculated using maximum composite likelihood. A) Alphaproteobacteria (Rhodobacterales; Rhizobiales; Sphingomonadales; Rhodospirillales); B) Gammaproteobacteria (Vibrionales; Enterobacteriales); C) Gammaproteobacteria (Alteromonadales; Pseudomonadales; Oceanospirillales); D) Bacteroidetes (Bacillales; Lactobacillales), tree continued to E) Actinobacteria (Actinomycetales), and Flavobacteria (Flavobacteriales). Alphaproteobacteria were rooted with *Bacillus weihenstephanensis*; Gammaproteobacteria were rooted with *Aquimarina latercula*; and Bacteroidetes, Actinobacteria, and Flavobacteria were rooted with *Vibrio parahaemolyticus*. Bootstrapping (n = 1000) was used to assess overall tree accuracy, and aligned nucleotide sequence gaps were treated by complete deletion. Bootstrap probabilities >50% are indicated at branch nodes. Sequences were compared against those published in the GenBank database. Phylogenies were reconstructed using various methods to confirm the overall tree topology. The scale bar represents two nucleotide (nt) substitutions per 100 nt positions, for A) and five substitutions per 100 nt positions for B), C), D), and E). GenBank accession numbers for reference strains are shown in parentheses, and site and corresponding particle filtrate information for *P. elisabethae*-associated isolates is included on the tree. MALDI-dereplicated isolates (2009 collections) are denoted by a single alphabetical letter and isolates selected for fermentation and natural product screening are denoted by the prefix RKVR (refer to Chapter IV). San Salvador isolates (2008) are denoted with an "SS" prefix or an "RKBH" prefix if cultivated by B. Haltli.

Flavobacteria (Figure 3.3). The most abundant genera were *Vibrio* (Gammaproteobacteria, 56 cultures), *Pseudoalteromonas* (Gammaproteobacteria, 33 cultures), *Ruegeria* (Alphaproteobacteria, 21 cultures), *Alteromonas* (Gammaproteobacteria, 17 cultures), *Bacillus* (Bacilli, 11 cultures), and a more diverse array of Actinobacteria (Figure 3.3).

San Salvador (2008)

In this investigation, isolation efforts to examine the cultivable bacterial community from *P. elisabethae* were more focused towards the isolation of Actinobacteria (for RKBH isolates) and the bacterial community from frozen coral homogenate (SS isolates). Collection efforts from frozen coral homogenate (SS isolates) resulted in a total of 17 isolates, which were dereplicated by RFLP to nine unique bacteria. Similarly, 31 bacteria were identified by RFLP dereplication of isolates obtained from fresh coral homogenate (RKBH isolates). With the exception of six isolates (SS9, SS17, RKBH-B001, RKBH-B103, RKBH-B005, RKBH-B110), all remaining isolates (n = 25) from the 2008 San Salvador collections were classified as Gram-positive. More specifically, these isolates represented a diverse array of Actinobacteria and Bacilli (Figure 3.3). It is well-known that Actinobacteria are prolific producers of secondary metabolites (Bérdy, 2005); yet little is known regarding the cultivable Actinobacterial communities from corals (Nithyanand and Pandian, 2009; Webster and Bourne, 2007; Zhang *et al*., 2008). Actinobacteria from the tissue of stony corals produce antibiotics (Nithyanand and Pandian, 2009) which target both Gram-negative and Gram-positive bacteria (Nithyanand *et al*., 2011). More recently, *Streptomyces* spp. from a black coral demonstrated moderate antimicrobial activity against marine bacteria and fungi (Zhang *et al*., 2012). Owing to the extensive reports on the biological activity of Actinobacteria and their recent discovery in corals, Actinobacteria isolated from *P.*

elisabethae may be a source of antimicrobial compounds. Similarly, members of the genus *Bacillus* are also common producers of antibiotic compounds, where approximately 800 antibiotic metabolites have been isolated (Wiese *et al*., 2009). Furthermore, *Bacillus* spp. have been implicated to play a role in coral protection, as marine-isolated *Bacillus* spp. exhibited antibacterial activity against several clinical pathogens and other marine bacteria (Shnit-Orland & Kushmaro, 2009). Therefore, *Bacillus* spp. may have roles in coral pathogen protection as producers of antibacterial compounds.

With respect to measures of culturable bacterial diversity for *P. elisabethae*, it is also important to note that the cultivation of bacteria from fresh coral homogenates gave rise to a greater diversity than bacteria isolated from frozen coral homogenate. This highlights the significance of inoculating microorganisms from fresh invertebrate homogenates and will be discussed subsequently. However, RKBH bacteria were isolated as the result of a more focused cultivation effort towards Actinobacteria. Actinobacteria were targeted as a consequence of the remarkable biological activities reported from natural products produced by this group (Bérdy, 2005). Consequently, homogenates of freshly collected coral and more diverse cultivation conditions were employed in subsequent investigations to obtain a more complete understanding of the culturable bacterial diversity in *P. elisabethae*.

Bimini (2009)

As a result of the low numbers of bacteria isolated from frozen *P. elisabethae* homogenate, a subsequent trial used fresh coral homogenate. In addition, diverse media conditions and the PFT were also employed in an attempt to increase the phylogenetic diversity of coral-associated bacterial isolates. A total of 686 bacteria were cultured from fresh *P. elisabethae* homogenate on

eight solid isolation media, where 16S rRNA gene sequences from 183 representative bacterial cultures were sequenced following MALDI-TOF MS dereplication. In this case, Gammaproteobacteria represented the largest group in the *P. elisabethae* culturable bacterial community and was dominated by *Vibrio* spp. (most abundant), *Alteromonas* spp., and *Pseudoalteromonas* spp. The abundance of these microbes may be indicative of their potential symbiotic roles within the coral holobiont. For example, both *Pseudoalteromonas* sp. and *Alteromonas* sp. appear to be probiotic bacteria in other invertebrates, including stony corals (Gil-Turnes *et al*., 1989; Kesarcodi-Watson *et al*., 2010; Tebben *et al*., 2011). Additionally, members of the genus *Pseudoalteromonas* are often associated with higher eukaryotes in marine environments and often produce natural products as reviewed elsewhere (Holmström and Kjelleberg, 1999). Conversely, bacteria of the genus *Vibrio* have been implicated as causative agents of coral disease (Ben-Haim *et al*., 1999). However, the abundance of *Vibrio* sp. from the *P. elisabethae*-associated culturable bacterial community should not be mistaken as a sign of compromised coral health, as literature has not defined a standard criterion for comparing *Vibrio* counts in healthy versus diseased corals (Daniels *et al*., 2011). Furthermore, abundant populations of *Vibrio* sp. have also been reported in other coral-associated bacterial cultivation studies (Brück *et al*., 2007; Koren and Rosenberg, 2006).

Alphaproteobacteria were also numerically dominant in these *P. elisabethae* samples and was dominated by *Ruegeria* sp. (21 cultures sequenced, Figure 3.3) and were most closely related to the type strain *Ruegeria mobilis* (NR_041454.1). Recently, *Ruegeria mobilis* was isolated from an endosymbiotic zooxanthellae *Symbiodinium* sp. within a reef-building coral. This isolate received attention for its possible role in coral larval metamorphosis by a mechanism not yet completely understood (Young, 2011). In addition to their potential roles in larval

development, *Ruegeria* sp. may also have biotechnological applications. For example, cyclic peptides have been isolated from sponge-associated *Ruegeria* sp., where moderate (25 – 50 µg/mL) and selective Gram-positive antimicrobial activity towards *Bacillus subtilis* was observed (Mitova *et al*., 2004). Therefore, *Ruegeria* sp. isolated from *P. elisabethae* may also be responsible for protecting the coral holobiont via the production of antimicrobial metabolites. Considering that *Ruegeria mobilis* was cultured in the present investigation in addition the the theory that *Ruegeria* sp. may be an alternative biosynthetic origin of the pseudopterosins, future screening of natural product gene clusters from cultured *Ruegeria* sp. associated with *P. elisabethae* may result in moving the scientific community one step further in solving the biosynthetic origin of the pseudopterosins.

Finally, Flavobacteria comprised a small yet significant portion of the *P. elisabethae* culturable community from fresh coral homogenate, dominated entirely by related *Aquimarina* sp. Specifically, these isolates were most closely related to the acroporid-derived *Flavobacteriaceae* sp. strain 04OKA-17-12 (MBIC08260). This coral was collected from the Okinawa prefecture in Japan, where the associated bacterium produced the carotenoids (3R)-saproxanthin and (3R, 3'R)-zeaxanthin. The former compound is rarely found in nature yet exhibited significant biological activity, including antioxidant and neuro-protective effects (Shindo *et al*., 2007). The antioxidative properties of carotenoids have attracted considerable interest due to their beneficial effects on human health (Berg *et al*., 2000). Within the coral holobiont, however, carotenoids may function to protect the coral from excessive solar radiation and provide antioxidant effects against free radicals (Britton, 1995; Vershinin, 1999). Hence, it is likely that the associated *Aquimarina* sp. within *P. elisabethae* also plays a symbiotic role within the coral holobiont.

The effect of the PFT on bacterial cultivation: Bimini (2009)

In general, the type and number of cultures were comparable between the two collection sites described in the previous section. However, *Vibrio nigripulchritudo*, *Sphingopyxis* sp., *Amphritea* sp., *Aquimarina* sp., and *Macrococcus* sp. were only cultured from Tuna Alley; conversely, *Arthrobacter* sp. and *Bacillus anthracis* were only cultured from Victory Reef. A higher number of yellow and maroon pigmented *Pseudoalteromonas* spp. were also cultured from Victory Reef (Table 3.3). Additionally, of seven potentially novel isolates, six were isolated from Tuna Alley (Table 3.4). This signifies that collection location may be an important variable in microbial cultivation. Furthermore, five of the seven potentially novel isolates were cultured from plating bacteria in the close physical presence of coral fragments as indicated by their cultivation on 51F and 104F screen filters, suggesting that the PFT is an effective tool to cultivate potentially novel bacteria. Although the PFT has been successfully implemented as a tool to detect slow-growing fungi (Hawksworth and Rossman, 1997), the PFT (refer to Figure 3.1) was employed here as a tool to cultivate bacteria which require the physical presence of coral tissue in an attempt to isolate tissue-associated bacteria. The majority of isolates were obtained from the particle filtrate (n = 324), followed by the 51 µm screen (n = 314), and the 104 µm screen (n = 48, Table 3.3).

To compare the species-level ($OTU_{0.03}$) diversity of the *P. elisabethae*-associated cultivable bacterial community between sites and from different particle sizes from the PFT, non-parametric estimates of richness (Chao1) and diversity (Shannon diversity index- *H'*, evenness-*E*) were calculated (Table 3.5). Overall, bacterial diversity as determined by the Shannon-Weiner index (*H'*) was slightly higher in Tuna Alley (average = 1.9) than Victory Reef

Table 3.3: The *P. elisabethae*-associated cultivable bacterial community representing sites, media, and the PFT. When more than one $OTU_{0.03}$ for a particular genus was obtained, isolates were recorded to the species level to clarify between congenera. "PF" refers to particle filtrate.

	Victory Reef			Tuna Alley			
Taxa	**PF**	**51F**	**104F**	**PF**	**51F**	**104F**	**Isolation Media**
Sphingopyxis sp.	-	-	-	2	1	-	MA
Vibrio nigripulchritudo	-	-	-	10	2	-	C, ASW, M3, MA
Vibrio sp.	1	2	-	1	-	-	MA, R2A
Bacillus aerophilus	1	-	-	1	-	-	C, MA
Micrococcus sp.	-	1	-	2	5	1	M3, C, R2A, MA
Ruegeria sp.	1	-	-	-	-	-	C
Janibacter terrae	1	-	-	1	-	-	MA, R2A
Pseudoalteromonas sp.	-	-	-	-	-	1	R2A
Pseudoalteromonas piscicidia (maroon)	-	3	-	-	1	-	MA
Photobacterium rosenbergii	2	-	-	5	1	1	PE, MA, ASW, R2A
Vibrio fortis/pelagia	3	1	-	19	2	4	Ps, MA, PE, C, R2A, ASW
Vibrio tubiashii	21	34	-	23	18	5	MA, M3, C, Ps, R2A, C, PE
Vibrio parahaemolyticus	49	60	-	39	37	11	MA, C, M3, R2A, PE, Ps, ASW
Amphritea sp.	-	-	-	1	1	-	M3
Pseudoidiomarina homiensis	-	1	-	-	-	-	MA
Janibacter anophelis	-	1	-	2	-	-	C, MA
Pseudoalteromonas piscicidia (yellow)	14	16	1	4	2	1	MA, M3, BG-11, C, ASW, Ps
Pseudomonas sp.	-	1	-	-	-	-	M3
Pseudoalteromonas phenolica	5	2	-	15	20	5	M3, C, R2A, MA, ASW
Pseudoalteromonas prydzensis	15	13	3	3	2	2	MA, C, R2A, BG-11, PE, ASW, Ps, M3
Bacillus weihenstephanensis	-	-	-	4	1	-	MA, M3, ASW, R2A, C
Ruegeria sp.	-	-	-	1	-	-	MA
Arthrobacter sp.	-	1	-	-	-	-	Ps
Bacillus anthracis	-	1	-	-	-	-	MA
Aquimarina sp.	-	-	-	2	3	-	ASW, MA, M3, C
Macrococcus sp.	-	-	-	3	2	-	C
Lysobacter sp.	-	-	-	-	1	1	C
Bacillus sp.	1	1	1	4	4	-	MA, M3, PE, ASW, C, BG-11
Ruegeria mobilis	20	26	1	15	5	1	M3, C, MA, BG-11, PE, R2A
Alteromonas sp.	15	16	1	12	21	8	M3, R2A, MA, BG-11, C, ASW, PE
Shewanella sp.	-	1	-	-	1	-	MA, M3
Paraliobacillus sp.	-	-	-	1	-	-	C
Kytococcus sp.	-	-	-	-	1	-	C
Marinilactibacillus sp.	-	-	-	-	1	-	R2A
Paracoccus sp.	-	-	-	-	1	-	ASW
Labrenzia sp.	-	-	-	4	-	-	M3, MA
Acinetobacter sp.	-	-	-	1	-	-	C
Total	149	181	7	175	133	41	
	Total site 1 cultures: 337			Total site 2 cultures: 349			Total number of isolates: 686

Table 3.4: Potentially novel isolates cultured from *P. elisabethae*. Site 1: Victory Reef, Site 2: Tuna Alley. "PF" refers to particle filtrate, 104F refers to isolates obtained from 104 µm screens, and 51F refers to isolates obtained from 51 µm screens.

Isolate	Nearest GenBank ID (% sequence identity)	No. of base pairs	Site and screen filter/PF	Isolation media
D10	*Pseudoalteromonas agarivorans* (NR_025509.1, 94%)	484	Site 2, 104F	R2A
V10	*Ruegeria pomeroyi* (NR_028727.1, **96%**)	408	Site 2, PF	MA
G25	*Lysobacter daejeonensis* (NR_043624.1, **95%**)	484	Site 2, 51F	C
E1	*Aquimarina intermedia* (NR_042444.1, **96%**)	1504	Site 2, 51F	SW
RKVR22	*Shewanella algae* (NR_028673.1, **96%**)	1530	Site 2, 51F	MA
RKVR31	*Paraliobacillus quinghaiensis* (NR_044411.1, **96%**)	1511	Site 2, PF	C
RKVR32	*Janibacter anophelis* (NR_043218.1, **96%**)	432	Site 1, 51F	C

Table 3.5: Diversity statistics of 16S rDNA sequence libraries generated from *P. elisabethae*-associated bacterial cultures. "PF" refers to particle filtrate, "51F" refers to bacteria isolated from the 51 µm screen, and "104F" refers to isolates obtained from the 104 µm screen from the PFT. Site 1: Victory Reef, Site 2: Tuna Alley.

Sequence library	**Total Seq.**	S_{obs} [a]	S_{est} [b]	H' [c]	E [d]
Site 1 PF	150	10	10	1.9	0.81
Site 1 51F	182	13	41	1.5	0.59
Site 1 104F	7	5	11	1.5	0.92
Site 2 PF	175	18	18.6	2.1	0.72
Site 2 51F	129	17	27.5	2.1	0.75
Site 2 104F	40	7	13	1.4	0.72

[a] Richness at species-level distance (D = 0.03) based on observed unique OTUs.
[b] Chao1 non-parametric richness estimate at species-level distance (D = 0.03).
[c] Shannon diversity index at species-level distance (D = 0.03); a higher number indicates greater diversity.
[d] Evenness index; a higher number indicates greater evenness.

(average = 1.6, Table 3.5). More specifically, Tuna Alley (particle filtrate) and Tuna Alley (51F) exhibited the greatest species level-bacterial diversity ($OTU_{0.03}$, 2.1 respectively), while the affiliation of bacteria with the particle filtrate suggests these bacteria are only loosely attached to the coral. In contrast, representatives of *Pseudoalteromonas* sp., *Vibrio/Photobacterium* sp., *Alteromonas* sp., *Bacillus* sp., *Ruegeria* sp., *Lysobacter* sp., and *Micrococcus* sp. were the only isolates retrieved from 104 µm screens (Table 3.3) and consequently (alongside Victory Reef (51F)) represented the lowest bacterial diversity (Table 3.5). However, the affiliation of these bacteria with larger mesh screens suggests these bacteria may have been tissue-associated.

Effect of media on bacterial cultivation: Bimini (2009)

As selection of isolation media is a key factor in influencing the cultivability and diversity of microorganisms (Zhang *et al*., 2012), this study used a wide variety of media containing different types and constitutions of nutrients. Three general isolation media were used, including MA, C media, and R2A, while a variety of specific media were used, including those which targeted Actinobacteria (M3), Cyanobacteria (BG-11), and *Pseudomonas* spp. (Ps media). In addition, PE medium was used with the rationale that uncultivable microbes may grow in pure culture if provided with cues from the natural environment (Webster and Hill, 2001) while ASW was used to enable the growth of potentially oligotrophic bacteria. Only 2009-plated coral homogenates were compared in a media examination owing to the success of cultivation and the diversity of the isolation media selected. As expected, isolation media affected the diversity and cultivability of *P. elisabethae*-associated bacteria. In particular, the highest numbers of morphologically unique colonies were obtained from the general isolation media MA and C, with the former isolation media also having been successful in retrieving large numbers of diverse culturable

bacteria from sponges (Nithyanand and Pandian, 2009; Webster *et al*., 2001) and octocorals (Brück *et al*., 2007). In contrast, despite the high number of bacteria isolated from R2A and M3 media, in general, only the most commonly obtained cultures (*e.g. Vibrio* sp. and *Pseudoalteromonas* sp.) were retrieved. Although Cyanobacteria phylotypes were abundant based on bTEFAP analyses (refer to Chapter II), no Cyanobacteria were cultivated despite the use of the selective medium BG-11. However, the inability to cultivate Cyanobacteria may be a methodological limitation. For example, although Cyanobacteria are photosynthetic organisms, plates were maintained in the dark for approximately one week during shipping back to Prince Edward Island. Additionally, precedence in the literature also suggests their cultivation may only be after specific criteria have been met, including light, enrichment, and reduction of oxidative stress in agar media (Choi and Noh, 2009; Morris *et al*., 2008; Webster and Hill, 2001).

In contrast to other investigators (Li *et al*., 2007; Olson *et al*., 2000), the addition of *P. elisabethae* extract to low nutrient media did not significantly increase the observed bacterial diversity, rather, fewer isolates were recovered on PE medium. However, *P. elisabethae* is the sole source of pseudopterosins, diterpene glycosides which have selective activity against Gram-positive pathogens (Ata *et al*., 2004; Correa *et al*., 2011). Consequently, it is likely that the addition of *P. elisabethae* extract to the medium negatively affected the isolation of Gram-positive bacteria. Consistent with Sipkema *et al*. (2011), however, fewer isolates were obtained from the oligotrophic medium ASW yet this medium was successful in isolating the potentially novel, slow-growing *Aquimarina* spp., which were rare isolates recovered in this investigation. The ability to cultivate *Aquimarina* spp. under oligotrophic conditions implies these bacteria have little nutritional requirements and may not be dependent on the host for providing nutrients.

Very few *Vibrio* spp. were cultured on ASW, which suggests these bacteria may be dependent on nutrients provided by the host for survival.

Unfortunately, this investigation was unsuccessful in obtaining the *Spongiobacter-Endozoicomonas* phylotypes or the candidate octocoral-specific bacterial phylum in pure culture (i.e. RKPE1 and RKPE23-related phylotypes respectively, refer to Chapter II). RKPE1 phylotypes were the most abundant phylotypes retrieved from our culture independent analysis of *P. elisabethae*-associated bacteria, while RKPE23-related phylotypes appeared to be exclusive to octocorals and may represent a novel phylum of bacteria. However, the nutritional requirements for the RKPE23-related phylotypes are unknown owing to their highly distant relationship (80% sequence identity to *Mycoplasma* sp.) to cultured representatives. Taken together, the results suggest that combining low and high nutrient media in addition to using fresh coral homogenate is essential for generating a higher cultivable bacterial diversity.

Many diverse factors influence the cultivability of marine microorganisms, including salinity, nutrient constituents, nutrient dilution, incubation conditions, and the presence of reactive oxygen species in agar media (Gray *et al*., 2011; Morris *et al*., 2008; Sharon and Rosenberg, 2008). More specifically, an inability to cultivate a higher amount of coral-associated bacteria may be explained by the following reasons: (1) cell damage caused by oxidative stress from cultivation media, (2) a lack of signaling molecules in microbiological media required for culture growth, and (3) an exhaustion of substrates during latter stages of the growth cycle (Bruns *et al*., 2002). Perhaps the most fascinating observation regarding the "uncultured" phenomenon is that many uncultured organisms, with the exception of the elusive *Candidatus* TM7 division for example (Hugenholtz *et al*., 2001), have very high 16S rRNA gene sequence

identities to well-studied cultured representatives. Strikingly, the 16S rRNA gene sequence of the proposed beetle-associated bacterium producing the antitumor polyketide pederin (Kellner *et al*., 2002a, b; Piel *et al*., 2004; Zimmerman *et al*., 2009) is 95% identical to the cultured reference strain *Pseudomonas otitidis* (NR_043289.1). Based on the cultivability of other *Pseudomonas* sp., this raises the question as to why a closely related bacterium can fail to grow *in vivo* equally well. What genetic or environmental factors prevent their cultivation? Scientists today require a total paradigm shift in order to understand how to successfully isolate environmental strains.

MALDI-TOF MS dereplication: Bimini (2009) isolates

To eliminate isolate redundancy in the bacterial library, MALDI-TOF MS was used as a tool to dereplicate coral-associated bacterial isolates. Representatives from each clade ('clade' defined here by a unique colour segment on the MALDI dendrogram) were sequenced in an attempt to determine an appropriate dendrogram distance-level cutoff. However, an appropriate dendrogram cutoff for UPGMA-clustered MALDI spectra to taxonomically distinguish between isolates has not been reported in the literature. Using this approach, 29 MALDI clades were obtained (Figure 3.4), representing 21 genera. Isolates were recorded to the species level to clarify between congenera (Figure 3.4). In general, there was good agreement between each member of the clade with corresponding 16S rRNA sequences. For example, *Ruegeria* spp. were conserved within the same clade on the dendrogram. Similarly, *Alteromonas macleodii* (NR_037127.1)-related isolates (n = 14 isolates sequenced) grouped at the base of the dendrogram, which were easily distinguished from the genetically related *Pseudoalteromonas* sp. The smallest sections on the dendrogram represented *Sphingopyxis* sp., *Janibacter* sp., and

Amphritea sp., and coincided with a smaller number of isolates sequenced. However, *Pseudoalteromonas* spp. and *Vibrio* spp. were not conserved within the same clade on the dendrogram respectively; rather, they were dispersed throughout (Figure 3.4). For example, sequences from isolates representing the largest clade of the dendrogram (yellow, Figure 3.4) demonstrated this clade contained a variety of *Vibrio* spp., including the closely related *Photobacterium rosenbergii*, *V. fortis/pelagia*, *V. shilonii*, *V. parahaemolyticus*, *V. harveyii*, and

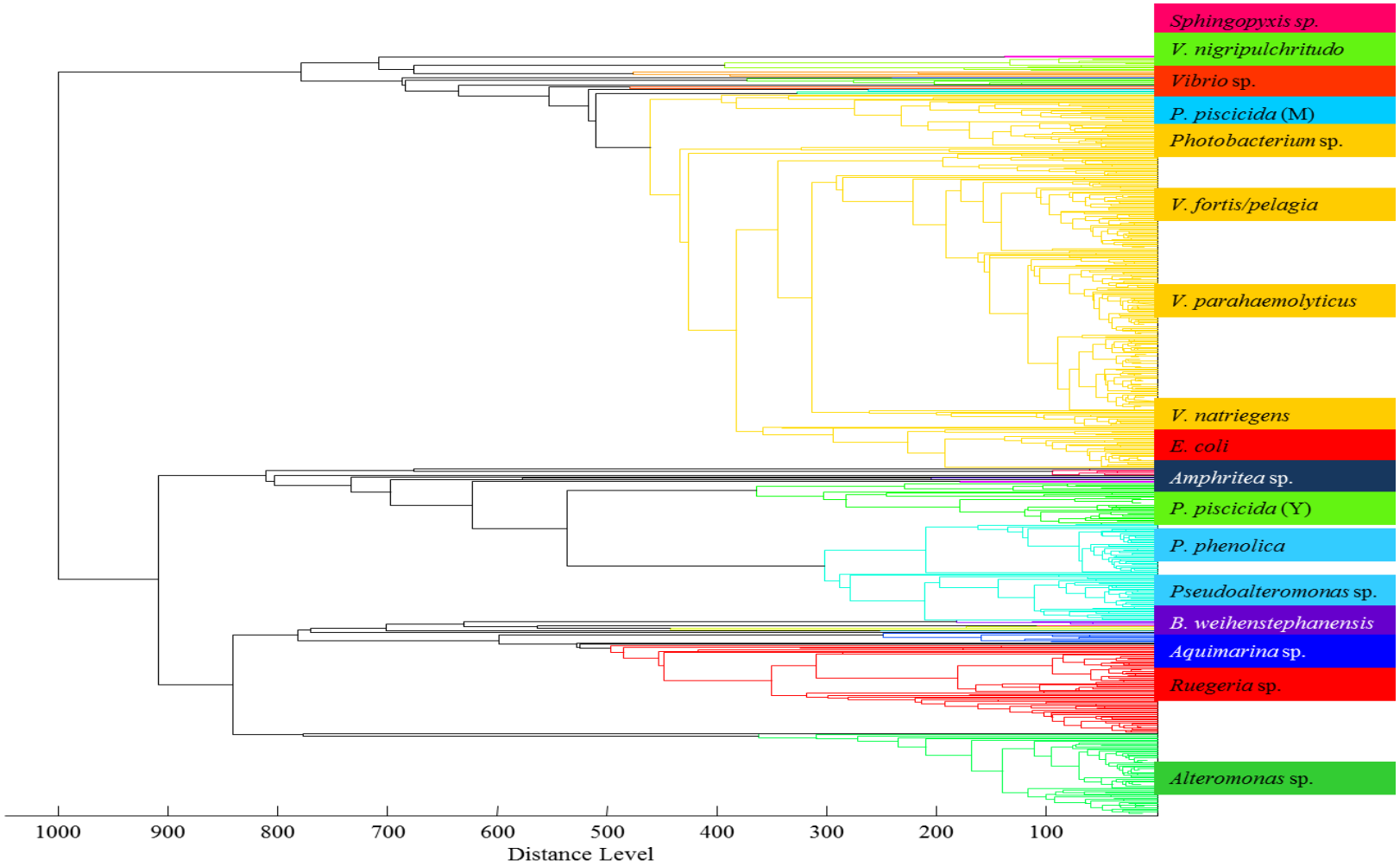


Figure 3.4: MALDI dendrogram of bacterial isolates from *P. elisabethae* clustered by UPGMA. 'M' refers to maroon pigmented isolate; 'Y' refers to yellow-pigmented isolate of *P. piscicida*. Phylogeny was inferred to the species level when necessary to distinguish between congenera.

V. natriegens. Similarly, phylogenetically related *Pseudoalteromonas* sp. did not cluster together in similar clades on the dendrogram. For example, the yellow and maroon-pigmented *Pseudoalteromonas piscicida* were closely related on the basis of 16S rDNA sequences, yet these grouped in entirely different clades on the dendrogram (Figure 3.4). Furthermore, as noted previously, difficulties in species differentiation of *Pseudoalteromonas* sp. by 16S rRNA gene sequencing necessitated nearly full length (~1,400 bp) sequencing in sponge associated *Pseudoalteromonas*; however, MALDI-TOF MS readily differentiated these species (Dieckmann *et al*., 2005).

MALDI-TOF MS will likely rely on sequencing to establish within-clade dendrogram cutoff values

Similar to Dieckmann *et al*. (2005), no clear dendrogram cutoff value could be established for cultivable bacterial isolates, as each clade was unique with respect to a dendrogram cutoff value. For example, isolate sequences were obtained for the uppermost and lowermost section of the *Alteromonas* clade (Figure 3.4). Sequencing results demonstrated that all sequences were related to *Alteromonas macleodii*, which corresponds to a distance cutoff of 300 – 400. Similarly, the dendrogram 'cutoff' for *Ruegeria* sp. could be defined at approximately 350, signifying that all isolates within the clade at a cutoff of 350 were similar when sequenced. In contrast, the *Pseudoalteromonas* clade (blue) was subdivided into two species based on 16S rRNA gene sequences, with the uppermost section of the clade more similar to *P. phenolica* while the lowermost section was most similar to a sediment-isolated *Pseudoalteromonas* sp. (FJ170036.1). As a consequence of these differences, it is likely that MALDI-TOF MS dereplication will continue to require isolate sequencing (Dieckmann *et al*., 2005). However, an in-house MALDI-

TOF MS spectral library complete with taxonomic information from isolate gene sequences has been established in the Kerr lab. This information can be used to guide future investigations in the Kerr lab to reduce sequencing effort.

Spatial and temporal comparison of *P. elisabethae*-associated bacterial isolates

Conserved between year (2008 and 2009), site (San Salvador, Victory Reef, and Tuna Alley) and homogenate type (fresh and frozen) were isolates closely related to *Bacillus* sp., and *Micrococcus* sp., while *Pseudomonas* sp. was conserved between collection year and between San Salvador and Victory Reef. Their consistent presence at different times and from different locations in the Bahamas suggests that these phylotypes may be key symbiotic members in *P. elisabethae*. *Pseudomonas* spp. have also been detected in other healthy coral-associated bacterial libraries (Koren and Rosenberg, 2006), and thus may form a natural part of the normal microbial fauna in corals. However, all *Vibrio* spp., *Ruegeria* spp., *Pseudoalteromonas* spp., *Alteromonas* spp., *Macrococcus* spp., *Photobacterium* spp., *Amphritea* spp., *Labrenzia* spp., *Shewanella* spp., and *Sphingopyxis* spp. were only retrieved from fresh (2009-collected) coral homogenates. Isolates belonging to the genera *Staphylococcus* spp., *Nocardiodes* spp., and *Streptomyces* spp. were unique to frozen coral homogenates.

It is likely that the consistent presence or abundance of select bacteria is attributed to their ease of cultivation on standard laboratory media, while the poor viability of bacteria from long-term stored, frozen coral homogenates may have reduced the types of bacteria which could be isolated on laboratory media. Not surprisingly, bacteria vary in their susceptibility to freezing (Haines, 1938), where Gram-positive bacteria are more resistant to freezing than Gram-negative bacteria, which may be attributed to the thinner peptidoglycan layer associated with the latter

(Miyamoto-Shinohara *et al*., 2008). Furthermore, spore-forming bacteria can withstand sub-zero temperatures, while more sensitive isolates can experience up to an 80% mortality rate upon rapid freezing (Haines, 1938). It is possible that the abundance of most Gram-negative bacteria was not reflected in isolation efforts from frozen coral homogenate as a consequence of storage at -80°C. For example, a significant reduction in the population of the foodborne pathogen *V. parahaemolyticus* was detected following five days of storage at -18°C (Vasudevan *et al*., 2002), which may explain the lack of *Vibrio* sp. from frozen coral homogenates despite their prevalence from isolation efforts using fresh coral homogenates. In the natural environment, *P. elisabethae*-associated bacteria are obviously not accustomed to freezing; therefore, it is likely that *P. elisabethae*-associated bacteria may not have evolved adaptations to survive freezing conditions. Although the importance of fresh coral homogenate is advocated in cultivation studies, it is also concluded that freezing coral homogenate prior to plating adds a unique benefit by increasing the phylogenetic diversity of bacteria, as rapidly-growing Gram-negative bacteria appear to be eliminated, permitting the isolation of low GC and Gram-positive bacteria on solid media.

It is acknowledged that bacteria from frozen coral homogenates were also collected a year prior and at a different collection site and may not be representative in all cases. However, the discrepancy in total numbers of cultures between fresh and frozen coral homogenates is not suspected to be a consequence of isolating bacteria from different collection sites or collection years and is believed to simply be a function of the cultivation procedures employed.

Comparison of the culturable and culture-independent bacterial community from *P. elisabethae*

It is beneficial to use a combination of culture-based and culture-independent methodologies to circumvent the limitations associated with each technique (Càrdenas *et al*., 2012) and thus provides a more complete understanding of the true bacterial diversity associated with samples. All isolate sequences from both collection years (2008 and 2009) were compared to *P. elisabethae* pyrosequencing libraries to provide an overview of the overlap of bacteria obtained between each method. When comparison of isolates to one pyrosequencing library was necessary, the sample 3E bTEFAP community was used as it was collected from the same site and collection year as 2009-isolated cultures, thus providing the most relevant comparison of the culture-independent to the culturable bacterial community. One would assume that bacteria identified by culture-independent techniques should overlap with bacterial isolates, yet cross-correlation of bTEFAP sequences to bacterial isolate sequences from *P. elisabethae* clearly demonstrates bacterial diversity is underrepresented by cultivation-based approaches (Table 3.6).

In total, 13 *P. elisabethae* pyrosequencing libraries were clustered with isolate sequences, resulting in 8,219 $OTU_{0.03}$s including singletons (4,985 $OTU_{0.03}$s excluding singletons, which may represent sequencing errors (Tedersoo *et al*., 2010)). From the combined dataset, 36 $OTU_{0.03}$s were common to both pyrosequences and isolate sequences, 32 $OTU_{0.03}$s were unique to cultures, and 8,150 pyrosequences were not represented by an isolate sequence, representing a 0.44% overlap (0.72% excluding pyrosequencing singletons) of cultures to pyrosequences from the *P. elisabethae*-associated bacterial library. A comparative overview of the cultivable community to the culture-independent bacterial community is presented in Figure 3.5.

Table 3.6: Comparison of the overlap of 16S rRNA gene sequences obtained from cultivation and from the sample 3E culture-independent (bTEFAP) data.

	bTEFAP	**Cultures**
No. of sequences	3781 dereplicated to 292 (at 97% sequence similarity)	685, de-replicated by MALDI and 206 sequenced
No. of bacterial phyla represented	11	4
Number of bacterial classes	22	5
Classes	Alphaproteobacteria (68%) Flavobacteria (21%) Cyanobacteria (4%) Gammaproteobacteria (4%)	Gammaproteobacteria (55%) Alphaproteobacteria (15%) Bacilli (15%) Actinobacteria (13%)
Most abundant genera	*Aquimarina* *Pelagibius* *Brevundimonas*	*Vibrio* *Pseudoalteromonas* *Ruegeria* *Bacillus*
Total number of genera	90	37

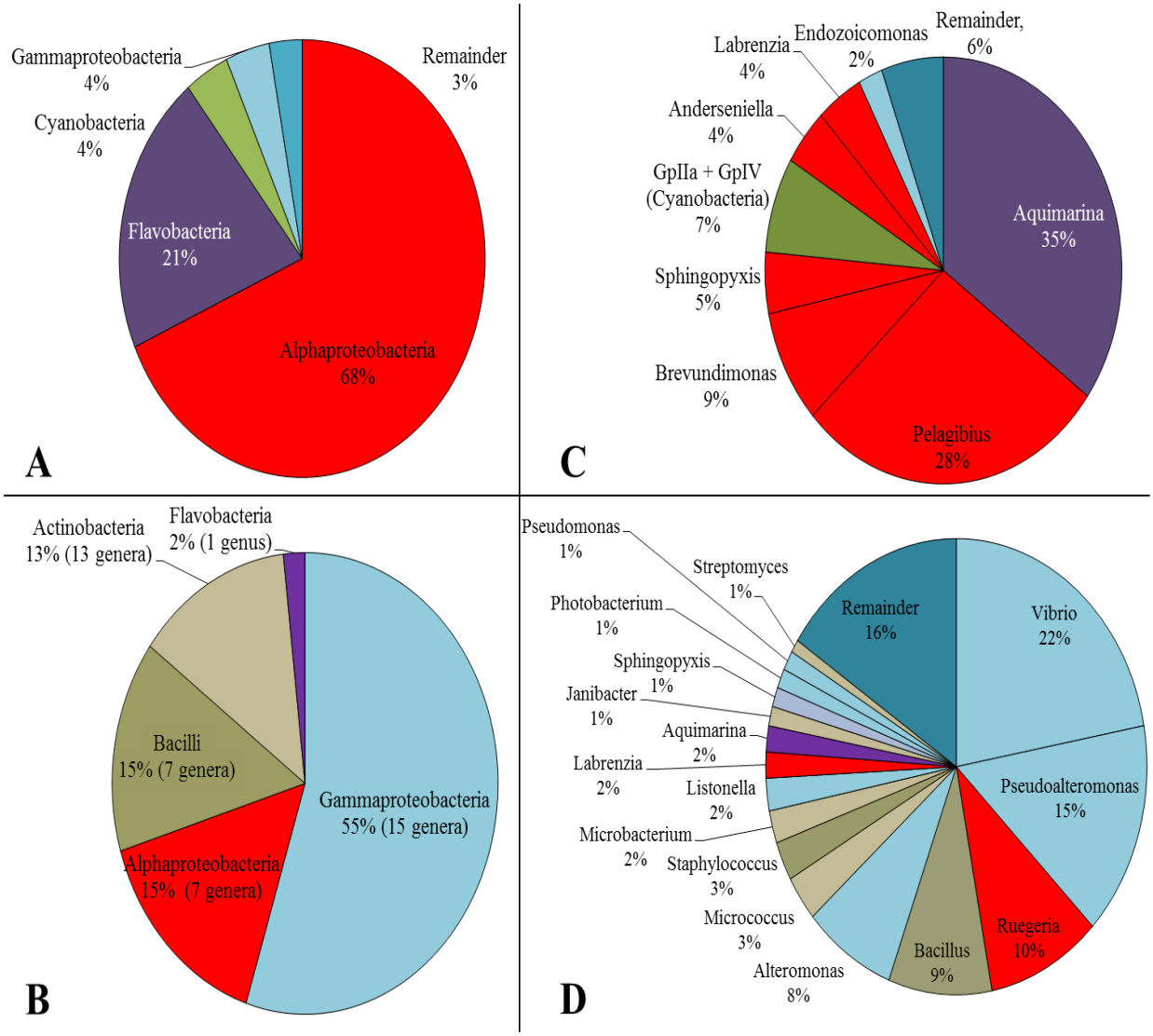


Figure 3.5: Composition of the culture-independent and cultivable *P. elisabethae* bacterial community. A) Culture independent community composition at the class level of coral sample 3E. B) Culture-dependent community composition at the class level of bacteria isolated from two *P. elisabethae* colonies. C) Culture-independent composition of *P. elisabethae* sample 3E at the genus level, and D) Culture-dependent composition of *P. elisabethae* isolates at the genus level.

Briefly, culture-independent analysis of sample 3E showed a predominance of Alphaproteobacteria, Flavobacteria, Cyanobacteria, and Gammaproteobacteria at the class level (Figure 3.5A). In particular, the sample 3E community was dominated by *Aquimarina* sp. and *Pelagibius* sp. phylotypes (Figure 3.5B). Among the culturable bacterial community; however, Gammaproteobacteria (55% of library), Alphaproteobacteria (15.3%), Bacilli (14.9%), and Actinobacteria (13.1%) were the most abundant classes (Table 3.6, Figure 3.5C), consisting of the genera *Vibrio*, *Pseudoalteromonas*, and *Alteromonas* (Figure 3.5D). In addition, highly abundant 16S rRNA gene sequences in culture libraries were often low in abundance or absent in all pyrosequencing libraries (Table 3.7). For example, only four isolate sequences overlapped with dominant phylotypes in the pyrosequencing library ("dominant" defined as $OTU_{0.03}$s in abundance of ≥1% in individual coral-associated bacterial libraries, classified as 'RKPE' phylotypes). This included the bTEFAP-generated phylotypes RKPE02, RKPE47, RKPE48, and RKPE168, which corresponded to the isolate sequences RKVR24 (*Aquimarina* sp.), RKVR10 (*Labrenzia* sp.), RKVR42 (*Pseudomonas* sp.), and RKVR35 (*Sphingopyxis* sp.) respectively (Table 3.7). Furthermore, the abundance of *Vibrio* sp. in culture collections was not represented in equal abundance by bTEFAP sequencing libraries. For example, of the 53 *Vibrio* spp. sequenced from cultures, only nine sequences were also found in all 13 PCR-generated *P. elisabethae* bTEFAP pyrosequencing libraries examined (refer to Table 2.1, Chapter II for descriptions). *Ruegeria* spp., *Pseudoalteromonas* spp., *Alteromonas* spp., and *Bacillus* spp. were also rarely observed in culture-independent sequence libraries (Table 3.7). For example, the most abundant *Pseudoalteromonas* sp. cultured (*e.g.* RKVR02-related isolates) did not have any representative $OTU_{0.03}$ when 13 representative pyrosequencing libraries were compared.

Table 3.7: Overlap of cultured bacteria to 13 *P. elisabethae*-associated bTEFAP libraries.

Sequences were clustered at $OTU_{0.03}$ and taxonomy was inferred by nearest GenBank identities.

No. of sequences in cluster	Representative Culture	No. of isolates	No. of bTEFAP sequences ($OTU_{0.03}$)	Genus	Representative bTEFAP sequence (≥1% of library)
6159	RKVR24	2	6157	*Aquimarina* sp.	RKPE02
346	RKVR10	3	343	*Labrenzia* sp.	RKPE47
333	RKVR42	2	331	*Pseudomonas* sp.	RKPE48
88	RKVR35	3	85	*Sphingopyxis* sp.	RKPE168
76	SS1	2	74	*Staphylococcus* sp.	
72	RKVR28	1	71	*Acinetobacter* sp.	
54	RKBH-B107	1	53	*Enterobacter* sp.	
49	D52	1	48	*Labrenzia sp.*	
38	V10	1	37	*Ruegeria sp.*	
28	RKVR37	3	25	*Bacillus* sp.	
24	RKVR18	11	13	*Bacillus* sp.	
16	RKVR15	3	13	*Photobacterium* sp.	
12	SS15	1	11	*Staphylococcus* sp.	
12	SS9	1	11	*Silicibacter* sp.	
18	RKVR03	8	10	*Pseudoalteromonas* sp.	
10	RKBH-B58	1	9	*Brevibacterium* sp.	
10	SS17	1	9	*Cohaesibacter* sp.	
10	RKVR22	2	8	*Shewanella* sp.	
26	RKVR16	20	6	*Ruegeria* sp.	
21	RKVR08	15	6	*Alteromonas* sp.	
14	RKVR17	8	6	*Micrococcus* sp.	
7	RKVR14	2	5	*Alteromonas* sp.	
6	RKBH-B56	1	5	*Staphylococcus* sp.	
20	RKVR21	16	4	*Vibrio* sp.	
24	RKVR19	21	3	*Vibrio* sp.	
12	RKVR05	9	3	*Pseudoalteromonas* sp.	
5	E3	2	3	Flavobacteria	
5	RKBH-B54	2	3	*Streptomyces* sp.	
18	RKVR01	16	2	*Vibrio* sp.	
3	F3	2	1	*Bacillus sp.*	
3	RKBH-B65	2	1	*Staphylococcus* sp.	
3	SS2	2	1	*Nocardioides* sp.	
2	RKBH-B114	1	1	*Bacillus* sp.	
2	RKBH-B103	1	1	*Roseomonas* sp.	
2	RKBH-B55	1	1	*Bacillus* sp.	

Consequently, the data presented herein confirms the power of complementing techniques to uncover the true bacterial diversity associated with marine invertebrates.

Literature precedence supports a lack of congruence between culture-dependent and culture-independent bacterial diversity

Although some isolate 16S rDNA sequences overlapped with the bTEFAP sequence community (Table 3.7), our results demonstrated <1% of bacteria from the *P. elisabethae* community were amenable to culturing. It is acknowledged that the sample size of cultivated isolates is smaller than the number of sequences generated by pyrosequencing, yet it is intriguing to question why some isolates did not have a culture-independent sequence representative. This lack of congruence between culture-dependent and culture-independent techniques is a trend which has been repeatedly documented in the literature (Galkiewicz *et al*., 2011; Gray *et al*., 2011; Jackson *et al*., 2012; Kellogg *et al* 2009; Kvennefors *et al*., 2012; Meron *et al*., 2011; Neulinger *et al*., 2008; Rohwer *et al*., 2001). For example, comparison of clone libraries to cultured isolates in the cold water deep-sea coral *Lophelia pertusa* reported no overlap between clone libraries and cultured isolates (Galkiewicz *et al*., 2011; Kellogg *et al*., 2009; Neulinger *et al*., 2008). Furthermore, Gray *et al*. (2011) found that *Vibrionaceae* were undetected in clone libraries of six deep sea alcyonacean octocorals yet were dominant in cultures. While it is well known that cultivation underestimates microbial diversity (Engelbrektston *et al*., 2010; Uthicke and McGuire, 2007), next generation sequencing technology is also not free from biases (Lee *et al*., 2012; Pinto and Raskin, 2012), which may also explain why some cultures in the *P. elisabethae* community were poorly represented by pyrosequencing when sequences were clustered at 97% sequence similarity (Table 3.7). For example, it is possible that some bacteria were present at

levels below the limit of bTEFAP detection, and increasing the coverage of bTEFAP may increase the likelihood of detecting these groups. Taken together, the results highlight the importance of complementing next generation sequencing with cultivation to obtain a more complete understanding of the true bacterial diversity associated with such samples as the alcyonacean octocoral examined in this study.

Inhibition of *P. elisabethae*-associated bacterial isolates by pseudopterosin G

Isolates were tested for their sensitivity to PsG with the objective of determining the antimicrobial effect of this natural product on the cultivability and growth of potential bacterial symbionts in *P. elisabethae* and to shed light on its plausible ecological role. The hypothesis which was tested was that Gram-positive bacteria would be more susceptible to PsG than Gram-negative bacteria based on prior evidence of PsG against clinically relevant microorganisms from Caribbean and Colombian-collected *P. elisabethae* (Ata *et al*., 2004; Correa *et al*., 2011). In this study, isolates growing on agar media were transferred to media containing either 0 µg/mL PsG, 5 µg/mL PsG, or 50 µg/mL PsG. Viability of isolates was checked and isolates were defined as 'sensitive' to PsG if their growth was compromised, but not completely inhibited, while isolates unable to grow in the presence of PsG were termed 'inhibited.' The addition of purified PsG to the media was capable of inhibiting or restricting the growth of several isolates. All isolates grew at 0 µg/mL PsG (Table 3.8). As expected, the majority of isolates affected by the addition of PsG to the media were Gram-positive, with 17 of 38 (44.7%) Gram-positive bacteria inhibited and seven sensitive (18.4%; indicated by a perispomeni in Table 3.8) at 50 µg/mL PsG. Additionally, nine isolates were inhibited and 14 were sensitive at 5 µg/mL PsG, revealing that PsG has broad spectrum activity against potential Gram-positive coral-associated bacteria. In contrast, only two

Gram-negative bacteria were inhibited and one Gram-negative bacterium was sensitive to the addition of 50 µg/mL PsG (7.9%). One Gram-negative isolate was sensitive and one Gram-negative isolate was inhibited by the addition of 5 µg/mL PsG. The bioactivity of PsG against Gram-positive bacteria may also be a factor in explaining why the majority of isolates and representative pyrosequences from *P. elisabethae* were Gram-negative.

The susceptibility of Gram-positive bacteria to PsG may be a consequence of their cell wall composition. For example, Gram-negative bacteria have an additional cell wall layer surrounding their membranes which is absent in Gram-positive bacteria, a feature which enables Gram-negative bacteria to prevent some antibiotics from crossing the membrane (Hopwood, 2007). The possible biological role of PsG in *P. elisabethae* may involve regulating the internal microbial community by preventing the overgrowth of potentially pathogenic Gram-positive bacteria as has recently been addressed elsewhere (Correa *et al*., 2012). Caution is advised when interpreting these results, as the localization of pseudopterosins in the host is not yet known. In addition, the visibly declined growth of some of the bacteria in the presence of pseudopterosins may imply that pseudopterosins may serve to regulate and reduce, not eliminate, bacteria residing within the host.

Salinity requirements of *P. elisabethae*-associated isolates

Although no clear term has been used for defining bacteria as "marine", the general consensus for using this definition is based on their requirement for sodium (Jensen and Fenical, 1994). For the purpose of this experiment, the goal in testing for salinity requirements was to add to the scientific understanding of those bacteria isolated from marine invertebrates which are dependent on seawater for growth (Jensen and Fenical, 1994). In the present study, isolates which required

or could grow with salt are indicated by a "yes" while isolates capable of growth without salt are indicated by a "no" (Table 3.8). As expected, the majority of isolates (57.1%) from *P. elisabethae* were considered "marine". Interestingly, "marine" bacteria defined here were far more likely to be Gram-negative bacteria, where 33/38 Gram-negative isolates (86.8%) were dependent on the addition of salt to the isolation media for growth in comparison to only 11/38 (28.9%) of Gram-positive bacteria (Table 3.8). This interesting trend, where Gram-positive organisms isolated from the marine environment are not dependent on salt (specifically sodium chloride) for growth has been reported previously (Jensen and Fenical, 1994). This may be explained by the fact that cellular respiration is dependent on Na^+ in some marine-isolated Gram-negative bacteria, while some marine-isolated Gram-positive bacteria lacking the respiration-driven Na^+ pump did not require sodium for the respiration (Ventosa *et al*., 1998). Salt tolerant bacteria have interesting industrial applications, particularly in providing the genetic machinery for salt and drought tolerance in agricultural crops, ultimately promoting crop growth in more saline soil (Ventosa *et al*., 1998). In addition, moderately halophilic bacteria provide an excellent model for the molecular study of the regulatory mechanisms which provides these bacteria with an ability to survive in a wide range of saline environments (Ventosa *et al*., 1998). However, it is important to note that the original isolation media (Table 3.1 and 3.2) all contained salt; therefore, it is difficult to comment on whether any exclusively "terrestrial" bacteria (isolates unable to grow in the presence of sodium chloride) were associated with the cultivable community of *P. elisabethae*.

Table 3.8: Salinity requirements and pseudopterosin G inhibition for *P. elisabethae*-associated isolates. A perispomeni (~) under 'salt required' refers to reduced visible growth of the isolate. ND: no data.

ID	Taxonomy	Salt required?	PsG 0 µg/mL growth	PsG 5 µg/mL growth	PsG 50 µg/mL growth
RKVR01	*Vibrio* sp.	Yes	✓	✓	✓
RKVR02	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR03	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR04	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR05	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR06	*Vibrio* sp.	Yes	✓	✓	✓
RKVR07	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR08	*Alteromonas* sp.	Yes	✓	✓	✓
RKVR09	*Alteromonas* sp.	Yes	✓	✓	✓
RKVR010	*Alteromonas* sp.	Yes	✓	✓	✓
RKVR011	*Janibacter* sp.	No	✓	✓	X
RKVR012	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR013	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR014	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR015	*Photobacterium* sp.	Yes	✓	✓	✓
RKVR016	*Ruegeria* sp.	Yes	✓	✓	✓
RKVR017	*Micrococcus* sp.	Yes	✓	✓	✓
RKVR018	*Bacillus* sp.	No	✓	X	X
RKVR019	*Vibrio* sp.	Yes	✓	✓	✓
RKVR020	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR021	*Vibrio* sp.	Yes	✓	✓	✓
RKVR022	*Shewanella* sp.	Yes	✓	✓	✓
RKVR023	*Alteromonas* sp.	Yes	✓	✓	✓
RKVR024	Flavobacteriaceae	Yes	✓	✓	X
RKVR025	Flavobacteriaceae	Yes	✓	✓	✓
RKVR026	*Macrococcus* sp.	Yes	✓	✓	✓
RKVR027	*Paracoccus* sp.	Yes	✓	✓	✓
RKVR028	*Marinilactibacillus* sp.	No	✓	✓	✓
RKVR029	*Micrococcus* sp.	No	✓	✓	✓
RKVR030	*Ruegeria* sp.	Yes	✓	✓	✓
RKVR031	*Paraliobacillus* sp.	~	✓	~	X

RKVR032	*Janibacter* sp.	~	✓	✓	✓
RKVR033	*Ruegeria* sp.	Yes	✓	✓	✓
RKVR034	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR035	*Sphingopyxis* sp.	Yes	✓	✓	✓
RKVR036	*Bacillus* sp.	Yes	✓	✓	✓
RKVR037	*Bacillus* sp.	Yes	✓	✓	✓
RKVR038	*Bacillus* sp.	Yes	✓	✓	✓
RKVR039	*Vibrio* sp.	Yes	✓	✓	✓
RKVR040	*Pseudoalteromonas* sp.	Yes	✓	✓	✓
RKVR041	*Pseudoidiomarina* sp.	Yes	✓	✓	✓
RKVR042	*Microbacterium* sp.	Yes	✓	✓	✓
RKVR043	*Vibrio* sp.	Yes	✓	✓	✓
RKBH B1	*Paracoccus* sp.	Yes	✓	✓	✓
RKBH B54	*Streptomyces* sp.	No	✓	X	X
RKBH B55	*Bacillus* sp.	No	✓	~	~
RKBH B56	*Staphylococcus* sp.	No	✓	~	~
RKBH B57	*Micrococcus* sp.	No	✓	✓	✓
RKBH B58	*Brevibacterium* sp.	Yes	✓	✓	✓
RKBH B62	*Nocardiopsis* sp.	No	✓	~	~
RKBH B65	*Staphylococcus* sp.	No	✓	✓	✓
RKBH B74	*Staphylococcus* sp.	~	✓	~	X
RKBH B75	*Microbacterium* sp.	No	✓	X	X
RKBH B80	*Exiguobacterium* sp.	Yes	✓	~	~
RKBH B86	*Pseudonocardia* sp.	Yes	✓	~	~
RKBH B100	*Microbacterium* sp.	No	✓	~	X
RKBH B101	Alphaproteobacteria	Yes	✓	✓	X
RKBH B102	*Rhodococcus* sp.	No	✓	~	X
RKBH B103	*Roseomonas* sp.	No	✓	✓	✓
RKBH B104	*Rhodococcus* sp.	No	✓	X	X
RKBH B105	*Salinicola* sp.	Yes	✓	✓	✓
RKBH B106	*Bacillus* sp.	No	✓	X	X
RKBH B107	*Enterobacter* sp.	No	✓	✓	✓
RKBH B108	*Planococcus* sp.	Yes	✓	✓	✓
RKBH B109	*Micrococcus* sp.	No	✓	~	~
RKBH B110	*Pseudomonas* sp.	Yes	✓	✓	✓
RKBH-B111	Alphaproteobacteria	No	✓	~	~
RKBH B113	*Gordonia* sp.	No	✓	~	X
RKBH B114	*Bacillus* sp.	~	✓	X	X
RKBH B115	*Kytococcus* sp.	No	✓	X	X

RKBH B116	*Bacillus* sp.	No	✓	~	X
RKBH B150	*Terribacillus* sp.	~	✓	~	X
RKBH B152	*Streptomyces* sp.	No	✓	X	X
RKBH B153	*Bacillus* sp.	No	✓	X	X
RKBH B154	ND	Yes	✓	✓	✓
RKBH B155	*Micrococcus* sp.	No	✓	~	~
RKBH B156	*Staphylococcus* sp.	No	✓	✓	✓

CONCLUSIONS AND PERSPECTIVES

The present study provided the first analysis of the cultivable bacterial community of *P. elisabethae* collected from the Bahamas. The use of unique isolation efforts in this investigation (PFT, selective media, dilution-to-extinction culturing, and plating bacteria from fresh and frozen coral homogenates) generated a diverse array of culturable bacteria. Although most of the isolates were closely related (≥ 97% sequence identity) to previously cultured bacteria, seven potentially novel bacteria were cultured. In light of the discovery of potentially novel isolates, it is suspected that the isolation conditions and the use of the PFT were significant factors in enhancing bacterial cultivation, therefore, PFT and select media specified in this investigation are recommended for future investigations of microbial cultivation from invertebrates. It is also concluded that plating fresh and frozen coral homogenates can shift the predominant microbial community from Gram-negative to Gram-positive bacteria respectively. This information can be used to guide future studies as a means to increase the biological diversity of cultivable prokaryotes. Additionally, this thesis showed the ecological significance of PsG (in addition to a recent report, Correa *et al*., 2012) which inhibits mainly Gram-positive bacteria associated with *P. elisabethae*.

Based on the notion that a high biological diversity directly translates to a high chemical diversity, the high cultivable bacterial diversity from *P. elisabethae* signifies that coral-associated bacteria are excellent targets to investigate for the production of bioactive natural products in subsequent analyses. Consistent with the great challenge facing microbiology today, however, less than one percent of *P. elisabethae*-associated cultures were amenable to culturing, a result which is in congruence with other investigations. Co-cultivation of *P. elisabethae*-associated bacterial isolates with other microorganisms may have increased the cultivability of

bacteria as has been suggested by other investigators (D'Onofrino *et al*., 2010; Pettit, 2009); other methods which can enhance microbial cultivation have been discussed elsewhere (Ben-Dov *et al*., 2009; Kaeberlein *et al*., 2002; Sipkema *et al*., 2011). Furthermore, future efforts will focus on culturing and biochemically characterizing isolates of intriguing groups of coral-associated bacteria, such as RKPE23-related phylotypes discussed previously, which share 80% sequence homology to *Mycoplasma* sp., to determine their metabolic capabilities and potential role(s) within their octocoral hosts. Regardless, the challenge facing microbiology today, which states that up to 99.8% of bacteria cannot be cultured on standard isolation media (Koren and Rosenberg, 2006) would benefit from a focused effort on A) understanding the factors which prevent cultivation and B) the development of novel culturing approaches. As a result, this may open new avenues for natural product drug discovery (Nithyanand and Pandian, 2009). Once the elusive factors which to-date prevent the cultivation of many microorganisms becomes available, this information can be used to increase our understanding of the microbiological world around us.

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CHAPTER IV

INVESTIGATION OF *PSEUDOPTEROGORGIA ELISABETHAE*-ASSOCIATED BACTERIAL ISOLATES FOR FERMENTATION, ANTIMICROBIAL SCREENING, AND CHEMICAL PROFILING

INTRODUCTION

With a large number of antibiotics targeting a variety of clinically devastating pathogens during the Golden Age, the world celebrated "the end of infectious disease." However, the process of natural selection desired to maintain the microbial world, and consequently microorganisms became progressively resistant to therapeutic treatments (Saleem *et al*., 2010). For example, highly resistant strains of the "superbug" methicillin-resistant *Staphylococcus aureus* (MRSA) were originally restricted to hospitals, yet are now widespread in the community (Lewis, 2012). In addition, more than 70% of hospital-acquired infections have become resistant to many antimicrobial agents, and today resistant bacteria exist in every country in the world (Coates *et al*., 2002).

Although the global market value for antimicrobial agents is approximately US $25 billion per year (Coates *et al*., 2002), knowledge that the use of an antimicrobial invariably selects for resistant microbes (Clardy *et al*., 2006) is discouraging to pharmaceutical companies, who are drawn towards treatments which can offer considerable commercial success, such as drugs consumed daily for lengthy periods of time (Baker *et al.*, 2007; Li and Vederas, 2009). However, of serious and immediate concern is the increasing crisis of microbial infectious diseases to which few effective antimicrobials are available (Zhang *et al*., 2005). Marine natural products provide a virtually untapped resource of structural diversity and diverse biological

activity, and therefore offer exceptional potential for the discovery of new antimicrobial agents (Baker *et al*., 2007).

Recent evidence suggests bacteria associated with marine invertebrates may be the true biosynthetic source of many promising marine natural products (Bunyajetpong *et al*., 2011; Bunyajetpong, 2011; Davidson *et al*., 2001; Gerwick *et al*., 2008; Kador *et al*., 2011; König *et al*., 2006; Sharp *et al*., 2007). Specifically, the octocoral *P. elisabethae* is a well-characterized source of antibacterial diterpenes (McCulloch *et al*., 2011). However, despite the growing body of literature suggesting bacteria are the true sources of many invertebrate-derived natural products as discussed above, *P. elisabethae*-associated bacteria have not been rigorously analyzed for natural product production. Given the well-established correlations between biological diversity and chemical diversity, it is rationalized that the associated bacterial community of *P. elisabethae* are a promising and sustainable source of novel natural products. The third aim of this research was to identify bacteria associated with *P. elisabethae* which are capable of producing novel secondary metabolites. As pointed out by Jensen and Fenical (1996), it is rationalized that bacteria will have become more adapted to the resources associated within the specific coral, and therefore it is unlikely that the same microorganisms or natural products will be discovered in other hosts (Jensen and Fenical, 1996).

Therefore, the ability of coral-associated bacteria to produce natural products was assessed by liquid chromatography/mass spectrometry following antimicrobial screening of bacterial extracts against a panel of clinically relevant pathogens. It is acknowledged that testing coral-associated bacteria against clinically relevant microorganisms may not be a true reflection of their antimicrobial or probiotic activity in their natural environment. However, studies have shown that clinically relevant biological activities are often translated into ecologically relevant

activities; for example, bacteria which demonstrate Gram positive activity in clinical isolates also act against Gram positive bacteria from within the host invertebrate (Hentschel *et al*., 2001).

MATERIALS AND METHODS

Small scale fermentation of *P. elisabethae*-associated bacteria

Bacterial isolates (n = 77) identified with the prefix "RKVR" or "RKBH" (refer to Table B2) were selected for fermentation and screening of fermentation extracts for antimicrobial activity. Isolates were chosen based on taxonomic affiliation via 16S rRNA gene sequences based on low gene sequence identity with published sequences and the likelihood that the genus could produce natural products based on literature precedence. Fermentation of all 77 bacteria were originally performed by J. MacAuley, N. Duncan, and N. McCarville. Reproducibility was assessed by V. Robertson where experiments were conducted in triplicate on two separate occasions. Although many of the selected *Pseudoalteromonas* spp. were genetically related, these isolates were selected because previous studies have indicated strains which were closely related exhibit different biochemical profiles and antibiotic sensitivity (Lampert *et al*., 2006); therefore, it is not implausible to suggest closely related bacteria may produce different natural products.

Bacteria selected for fermentation were obtained from 50% glycerol stocks stored at -80 °C and were plated immediately on Marine Agar 2216 (MA, BD Difco™) plates for two to seven days, until significant growth was observed. Any isolate which was visibly contaminated was subcultured to purity on fresh MA plates. To generate a sufficient biomass for small scale extraction, pure cultures were transferred using a sterile applicator in a laminar flow hood to 7

mL of seed media (Marine Broth, MB, BD Difco™) contained in 25 mm x 150 mm borosilicate glass culture tubes (VWR International, Mississauga, ON). Seed media was prepared according to the manufacturer's instructions. Three to five glass beads were added to each tube when filamentous bacteria were cultured to improve broth mixing and aeration. All tubes were capped with a plastic enclosure, autoclaved (121°C for 20 min), and cooled to room temperature. Tubes were incubated at 30°C and cultures were shaken at 200 rpm on a rotary shaker (Innova, New Brunswick Scientific) for two to seven days depending on growth in seed media. Bacteria were grown in seed media until confluent growth was observed. Following confluent growth, 100 µL (or 1 mL for mF/2 media) of cultures in seed media was used to inoculate 10 mL of 10 sterile fermentation media chosen for the induction of secondary metabolite production (Table 4.1). Media blanks (absence of bacterial inoculates) were incubated and extracted as a control. In the case of filamentous bacteria, wide bore pipette tips were used to ensure a sufficient biomass was transferred. Cells were harvested following three to seven days of growth, again depending on the observable growth of bacteria during fermentation. Following inoculation, 50% glycerol stocks were prepared from seed media in a sterile cryovial, mixed well, and stored at -80°C. Fermentations were assessed for purity by streaking 20 µL at each step on sterile MA plates and were incubated at 30°C. Following initial bioactivity screening of bacterial fermentation extracts, fermentations for 18 of the most active bacteria (defined by ≥80% inhibition against any of the tested clinical isolates) were performed in duplicate to ensure reproducibility.

Table 4.1: Fermentation media used in the cultivation of *P. elisabethae*-associated bacteria.

Medium	Medium Name	Rationale	Ingredients
M1	modified MB (mMB)	A general fermentation medium modified from MB through the addition of Instant Ocean as a salt source.	5 g/L BD Bacto Peptone, 1 g/L Yeast Extract (EMD), 0.1 g/L Ferric Citrate (Aldrich); 18 g/L Instant Ocean; Nanopure H_2O to 1L; pH 7.6 ± 0.2
M2	Terrestrial Broth (TrB)	Used as a medium to determine if secondary metabolite production can be stimulated by the absence of salt.	5 g/L BD Bacto Peptone, 1 g/L Yeast Extract (EMD), 0.1 g/L Ferric Citrate (Aldrich); Nanopure H_2O to 1L; pH 7.6 ± 0.2
M3	mMB + 0.25 g/L mevalonolactone (MEV)	Mevanolactone is a growth factor in some bacteria; added as a precursor for terpene biosynthesis.	5 g/L BD Bacto Peptone, 1 g/L Yeast Extract (EMD), 0.1 g/L Ferric Citrate (Aldrich); 18 g/L Instant Ocean; Nanopure H_2O to 1L; pH 7.6 ± 0.2; Added post-sterilization: 2.5 mL of 0.25 g of mevalonolactone dissolved in 2.5 mL 95% ethanol
M4	mMB + 50 mg/L pravastatin (PVS)	Pravastatin added as a bacterial growth promoting agent	5 g/L BD Bacto Peptone, 1 g/L Yeast Extract (EMD), 0.1 g/L Ferric Citrate (Aldrich); 18 g/L Instant Ocean; Nanopure H_2O to 1L; pH 7.6 ± 0.2; Added post-sterilization: 2.5 mL of 0.25 g of mevalonolactone dissolved in 2.5 mL 95% ethanol
M5	Trypticase Soy Broth (TSB)	Used as a general purpose medium, notably for non-sporulating organisms.	BD Difco premix: 17 g/L Pancreatic Digest of Casein; 3 g/L Papaic digest of soybean meal; 18 g/L Instant Ocean; 2.5 g/L K_2HPO_4; 2.5 g/L Dextrose; Nanopure H_2O to 1L
M6	BFM3	Selected owing to the production of five new polyketides from a fermentation of *Streptomyces* sp. (Graziani *et al*., 2005); modified to include Instant Ocean.	0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.5 g/L KCl; 3 g/L K_2HPO_4; 18 g/L Instant Ocean; 0.4 g/L Agar; 12 g/L Glycerol; 5 g/L Soy Peptone; Nanopure H_2O, to 1L; pH 7.0 ± 0.2
M7	Benzoate Minimal Salt (BMS)	Used as a cultivation medium for microorganisms which can use benzoate as a carbon source (ex. *Pseudomonas* sp.).	0.2 g/L Citric acid sodium salt; 10 g/L K_2HPO_4; 0.2 g/L $MgSO_4 \cdot 7H_2O$; 3 g/L $NaNH_4HPO_4 \cdot 7H_2O$; Nanopure H_2O to 975 mL; Instant ocean 18 g/L; pH 7.0 ± 0.2; Added post-sterilization: 25 mL of Sodium Benzoate (2.5 g dissolved in 25 mL Nanopure H_2O, filter sterilized)
M8	Modified mF/2 (mF/2)	Nutrient reduction of modified F media.	1 mL $NaNO_3$ stock solution, 1 mL NaH_2PO_4 stock solution, 1 mL Trace Metals solution; 0.5 mL Vitamin Stock solution; 0.25 g Proflo Oil; 0.09 g L-fucose; 0.09 g D-xylose; 0.09 L-arabinose; 0.09 g L-arabinose; 0.09 g D-glucosamine; 0.09 g N-acetyl-D-glucosamine; 0.30 g Difco Cas Amino Acids; 18 g/L Instant Ocean; Nanopure H_2O to 1L; pH 7.5 ± 0.2. NaH_2PO_4 Stock solution: (5g NaH_2PO_4 in 1L Nanopure H_2O). Trace metals stock solution: 4.36 g Na_2EDTA, 3.15 g $FeCl_3 \cdot 6H_2O$, 1.0 mL Primary trace metals, Nanopure H_2O to 1L. Primary Trace Metals Stock Solution (added post-sterilization): 1 g $CuSO_4 \cdot 5H_2O$, 2.2 g $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, 18 g $MnCl_2 \cdot 4H_2O$, 0.63 g $NaMoO_4 \cdot 2H_2O$; Nanopure H_2O to 100 mL. Vitamin Stock Solution (added post-sterilization): 10 mL of 0.1 mg/mL solution of Biotin, 1 mL of 1 mg/mL solution of Vitamin B12, 2 g Thiamine HCl.
M9	PE aqueous media (PEAq)	The addition of invertebrate extract has been demonstrated to increase the number of novel cultivated isolates in sponges (Webster *et al*., 2001).	500 mL of ground PE (100 g of ground *P. elisabethae* tissue was boiled and extracted with MeOH for 30 min in 1 L Nanopure dH_2O containing 18 g/L instant ocean); 9 g/L Instant Ocean; 1 g/L Yeast Extract; 1 g/L Dextrose; Nanopure H_2O to 1L; pH 7.5 ± 0.2
M11	mMB + HP-20	Added to the fermentation media to adsorb secondary metabolites and to prevent feedback inhibition during fermentation.	5 g/L peptone; 1 g/L Yeast Extract; 0.1 g/L Ferric Citrate; 18 g/L Instant Ocean; Nanopure H_2O to 1L; 10% Diaion® HP-20 w/v (1 g into 10mL)
M12	modified F media (mF)	A medium used to culture bacteria which may be dependent on symbiosis with the algal symbiont *Symbiodinium* sp. in *P. elisabethae*.	2 mL $NaNO_3$ stock solution (see mF/2); 2 mL NaH_2PO_4 stock solution (see mF/2); 1 mL Trace metals stock solution (see mF/2); 0.5 g Proflo Oil; 0.2 g L-fucose; 0.2 g D-xylose; 0.2 g L-arabinose; 0.2 g D-glucosamine; 0.2 g N-acetyl-D-glucosamine; 0.6 g Difco Cas Amino Acids; 18 g/L Instant Ocean; Nanopure H_2O to 1L; pH 7.5 ± 0.2

Extraction of secondary metabolites

Cultures were removed from the rotary shaker and transferred to 50 mL centrifuge tubes. Activated HP-20 (10% w/v) was added to all cultures except M11, and cultures were shaken at 250 rpm for one hour at room temperature. Resins and cells were pelleted by centrifugation for 7 min at 4000 rpm, and supernatants were carefully removed. To wash the resins, 30 mL of MilliQ® H_2O was added to each tube. Tubes were shaken on a rotary shaker at 250 rpm for 30 minutes, centrifuged as above, and the supernatant was removed. Next, 30 mL of 30% MeOH in H_2O was added, samples were shaken at 250 rpm (30 min), and the solvent mixture was removed. Finally, organic metabolites were removed from the HP-20 resin by adding 7 mL of 100% MeOH. The resins were shaken for 30 minutes, and MeOH was removed and transferred to a pre-weighed scintillation vial. The resins were re-extracted with 100% MeOH and shaken/transferred as described above, and extracts were dried using a GeneVac® evaporating system. Dried extracts were re-suspended in H_2O and extracts were partitioned using an equivalent amount of EtOAc. EtOAc fractions were dried and were defatted with hexane by partitioning with a 1:1 volume of hexane and ACN. ACN extracts were analyzed by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UPLC/HRMS) in addition to testing for antimicrobial activities.

Antimicrobial assay

Extracts obtained from fermentations were assessed for the production of antibiotics against five human pathogenic microorganisms (Table 4.2). Cation Adjusted Mueller Hinton Broth, CAMHB, Becton-Dickinson and Company) was used to culture bacterial strains and Sabouraud dextrose broth (SD) was used to culture *Candida albicans*; stock solutions were

Table 4.2: List of clinical isolates investigated from *P. elisabethae*-associated bacterial isolates.

Pathogen	**Affiliation**	**ATCC/Strain No.**	**Positive control**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Gram positive	ATCC 33591	Vancomycin
Vancomycin-resistant *Enterococcus faecalis* (VRE)	Gram positive	379*	Rifampicin
Pseudomonas aeruginosa (PA)	Gram negative	ATCC 14210	Gentamicin
Proteus vulgaris (PrV)	Gram negative	ATCC 12454	Ciprofloxacin
Candida albicans (CA)	Yeast	ATCC 14035	Nystatin

*Singh *et al.*, 2000.

prepared as outlined previously (Correa *et al.,* 2011). M. Lanteigne was responsible for performing all bioassay experiements.

Biological activities of fermentation extracts were tested using the broth microdilution method in 96 well microtiter plates as outlined previously (Correa *et al.,* 2011). To each assay plate, three vehicle controls (blank control) were added, consisting of 2% DMSO (total volume) and the appropriate media. Three organism controls (negative control) containing the pathogen in 2% DMSO were also included in each assay plate. Finally, the antibiotic control (positive control) was added, along with the pathogen, as a dilution series in 2% DMSO (final volume) with one dilution per well. Pathogens were adjusted to obtain a final cell count of 5×10^5 CFUs/mL. To prepare the assay plate, 10 µL of the test compound extract (prepared in 20% DMSO) were added into respective wells on the assay plate. In fermentation trial 1, extracts were not weighed, yet were transferred to the assay plate at 0.10X (*e.g.* 1.4 mL from a total fermentation volume of 14 mL was transferred and dried in assay plates). These extracts were tested undiluted (0.10X), at ½, ¼, and $^1/_{16}$. The optical density was measured at an absorbance of 600 nm (OD_{600}) at time 0 ($T_{initial}$) using a spectrophotometric BioTek® Synergy HT microplate reader (Vermont, USA). The plate was incubated at 37°C for 22 hours, and the final OD_{600} reading (T_{final}) was obtained. To determine the antimicrobial activities of the extracts or compounds, the percent inhibition was calculated for each measurement, taking into account medium controls without inocula and media controls with inocula. Antibiotic controls were also performed in serial dilutions to control for variances in pathogen sensitivity (Correa *et al.,* 2011). Half maximal inhibitory concentrations (IC_{50}s) were calculated by plotting concentration (*x*) versus percent inhibition (*y*) and locating at which point *x* crosses *y* at 50%. Antimicrobial activities were initially tested using all 77 de-replicated isolates (listed with the prefix "RKVR"

or “RKBH” in Table B2). However, to confirm reproducibility and to prioritize the workload, fermentations were repeated by selecting 18 of the most bioactive (defined as ≥80% inhibition against any clinical isolate) fermentation extracts at a 10X concentration against clinical pathogens following the first round of bioactivity screening. For fermentation trial 2, extracts were tested against pathogens at 125 µg/mL. Finally, serial dilutions of RKBH-B54 fermentation extracts were tested against pathogens at the following concentrations: 125, 63, 31, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/mL.

Chemical analysis of bioactive extracts by UPLC/HRMS

All bioactive fractions and extracts were analyzed by UPLC/HRMS using electrospray ionization (ESI). UPLC (Thermo Scientific, Accela) was equipped with a photodiode array (PDA) detector (Thermo Scientific, Accela) to identify compounds which absorb UV light, an electron light scattering detector (ELSD; Sedere, Sedex LT-ELSD model 80LT), and a high resolution mass spectrometer (Thermo Scientific, Exactive plus Orbitrap) to accurately determine the exact molecular formula through exact mass measurements. UPLC/HRMS was performed in positive ion mode using a standard gradient from 5% ACN in H_2O (0.1% formic acid in both eluents) to 100% ACN in a 10 minute run. Extracts were prepared in MeOH to a concentration of 500 µg/mL and reserpine (25 µg/ mL) was used as a standard for each set of samples. MeOH blanks were also run in each batch of samples. Chromatographic and spectral profiles were compared to media blank extract profiles to determine which compounds were produced as a result of bacterial metabolism. Pseudomolecular ions identified from the MS spectra were searched in natural product databases (Antibase 2007, SciFinder) to identify the putative novelty of the detected compounds. Antibase 2007 searches were performed by

searching pseudomolecular ions in positive mode ESI searching either proton adducts $[M+H]^+$ or sodium adducts $[M+Na]^+$. Deviations of experimental values from proposed molecular formulae values are recorded as δ (in ppm).

Large scale fermentation and extraction of RKBH-B54

Streptomyces sp. RKBH-B54 (nearest GenBank representative, *S. sampsonii* ATCC 25495, 99% sequence identity) was isolated and identified by B. Haltli from *P. elisabethae* on R2A media (BD Difco™, Mississauga, ON) based on partial 16S rRNA gene sequencing (refer to Chapter III). RKBH-B54 strongly and routinely inhibited the growth of the yeast *C. albicans*, and therefore large scale fermentation (4L) was performed in an attempt to isolate and identify the antifungal compound(s). A small scale fermentation and extraction (10 mL x 20 tubes = 200 mL total) was performed in parallel to the large scale extraction to ensure biological activity was retained when the isolate was fermented at a larger volume, as studies have shown that feedback inhibition of secondary metabolites can increase when larger fermentation batch sizes are used (Xu *et al*., 1999).

RKBH-B54 was streaked for purity on MA and was transferred using a sterile applicator to 10 mL of MB (seed media) containing five glass beads. Cultures were grown at 30°C for 72 hours in seed and were shaken at 200 rpm on a rotary shaker. A small aliquot of sample growing in seed was used to streak for purity on MA. The remainder of RKBH-B54 growing in seed was transferred using a sterile pipette into 50 mL of sterile MB in a 250 mL flask, which was shaken at 250 rpm for 24 hours at 30°C. Cultures were again assessed for purity by streaking on agar plates, and 12 mL each of second stage media was transferred to 1L each of autoclaved BFM3 into four - four liter Fernbachs covered by sterile milk filters and nitrocellulose membranes.

Following five days of growth in BFM3, 10% HP-20 resin (w/v) was added, and samples were shaken (250 rpm) at room temperature for one hour. Fermentation mixtures were transferred to 250 mL centrifuge bottles, filling no more than 200 mL in each centrifuge. Samples were centrifuged for 10 min at 10,000 rpm (Beckman Coulter Allegra 25R centrifuge), and the supernatant was removed. Resins were washed three times using 200 mL volumes of MilliQ® H_2O and were subsequently washed twice with 200 mL of 20% MeOH. Resins were extracted three times using 200 mL of MeOH to elute adsorbed solutes, the solvent was dried using a rotary evaporator, and the mass of dried extracts was obtained.

Bioassay guided purification of RKBH-B54 extracts

MeOH extracts of RKBH-B54 were dissolved in 1L of MilliQ® H_2O to a concentration of 1 g extract per L H_2O which was transferred to a 4L Fernbach containing 5% each (w/v) of two moderately polar adsorbent resins, activated Diaion® HP-20 and Amberlite® XAD-7. Making a slurry of the resins, the broth was filtered over the resins via vacuum filtration, using 1L each of increasing concentrations of organic solvents: F1, H_2O; F2, 80:20 H_2O:MeOH; F3, 50:50 H_2O:MeOH; and F4: 100% MeOH. All fractions were evaporated under reduced pressure using a rotary evaporator. F4 was subjected to solid phase extraction (SPE) using a C18 Sepak cartridge (Waters, Milford, MA). The sample was loaded onto the column and 15 mL of each of 6 solvent mixtures of decreasing polarity were used to elute compounds into pre-weighed scintillation vials: F1, 9:1 H_2O:MeOH; F2, 50:50 H_2O: MeOH; F3, 2:8 H_2O:MeOH; F4, 100% EtOH; F5, 100% acetone; and F6, 1:1 DCM:MeOH. Each fraction was concentrated *in vacuo* using a rotary evaporator, analyzed by UPLC/HRMS, and resubmitted for biological activity screening against *C. albicans*.

Chromatographic separation of the antifungal compound produced by RKBH-B54 using semi-preparative reverse-phased HPLC

Semi-preparative reverse-phased (RP)-HPLC of the bioactive C18 Sepak F3 was performed in an attempt to purify the antifungal compound(s) from the mixture. The sensitivity of the antifungal compound(s) to probable degradation was known prior to separation owing to a previous fermentation and RP-HPLC purification attempt (data not shown); therefore, caution was used by storing approximately half of the fraction F3 at -80°C (divided into "Trial 1" and "Trial 2" respectively). In Trial 1, C18-SPE F3 (~11 mg) was re-suspended to 10 mg/mL in H_2O:MeOH (1:3), sonicated, and centrifuged to remove excess particulate material. Fractions were monitored and collected by RP-HPLC on a Finnigan Surveyor LC Plus pump attached to a PDA plus detector (Thermo Electron Corp) and a Sedex 60 LT (Sedere) ELSD. UV was monitored at 220, 254, and 325 nm using ChromQuest (v. 4.2.34). The mobile phases were H_2O + 0.1% TFA (A) and ACN (B). A C18-semi preparative column (Phenomenex Gemini®, 250 x 10 mm, 5 µm) was used with the following elution conditions: $t_{0\ min}$ = 50%B, $t_{3\ min}$ =80%B; $t_{13\ min}$ = 80%B; $t_{13.01\ min}$ = 100% B; $t_{23\ min}$ = 100% B; $t_{25\ min}$ = 50% B. A 90 µL volume was injected onto the column with a flow rate of 2.5 mL/min. All solvents used in the purification were chromatography grade.

To purify the remainder of RKBH-B54 C18 Sepak F3 (8.32 mg) for Trial 2, the sample was removed from storage at -80°C, resuspended in H_2O:MeOH (1:3), and prepared as described above. Fractions were monitored and collected by RP-HPLC on a Waters HPLC (Milford, MA, USA) equipped with an ELS detector (Waters 2424), Binary LC pump, UV/Vis detector, and a fraction collector using Fractionlynx and Mass Lynx (v. 4.1) software. UV was monitored at 280 and 325 nm, using the same semi-preparative C18 column as in Trial 1. Similarly, H_2O + 0.1%

TFA (A) and ACN (B) were used as the mobile phase with a flow rate of 3 mL/min and injection volume of 100 µL. Separation was performed using the following conditions similar to those described from Jakobi *et al*. (1996): $t_{0\ min}$ = 50%B, $t_{24\ min}$ = 50%B; $t_{25\ min}$ = 100%B; $t_{31\ min}$ = 50%B; $t_{40\ min}$ = 50%B. Fractions were collected in clean glass tubes and dried under reduced pressure. Following separation of the entire amount of C18-SPE F3, a second HPLC separation on HPLC purified F3 was performed by applying a linear gradient from 10% to 60% ACN over 40 minutes.

RESULTS AND DISCUSSION

Antimicrobial activity of bacterial fermentation extracts

The aim of this investigation was to assess the ability of *P. elisabethae*-associated bacteria to produce natural products, as measured by the ability of bacterial fermentation extracts to show antimicrobial activity against a panel of clinically significant pathogens. Results from initial screening endeavors from 77 bacteria revealed that the majority of fermentation extracts were active against Gram positive pathogens, where 21 fermentation extracts were strongly and reproducibly active against MRSA and 23 fermentation extracts were strongly and reproducibly active against VRE (Table 4.3). Conversely, less activity was observed against Gram negative bacteria, with six extracts active against *P. vulgaris* and no isolates active against *P. aeruginosa* at 10X concentration (Table 4.3). This concentration was equivalent to testing an extract volume of 1.4 mL and was used as a means to more rapidly determine antimicrobial activity. Subsequent extracts were tested at a minimum of 125 µg/mL. In most cases, bioactivity against *P. vulgaris* was generally observed when bacteria were fermented in TSB (Table 4.3). The lack of antimicrobial activity against *P. aeruginosa* could suggest the coral specifically hosts bacteria which are incapable of competing with a potentially vital member of the coral holobiont. Only one coral-associated bacterial fermentation extract (*Streptomyces* sp. RKBH-B54) was capable of inhibiting the yeast *C. albicans* (Table 4.3).

The influence of fermentation media on antimicrobial production

Media selection is often a fundamental component in the success of microbial natural product production. Remarkably, features such as different media and minor variations of nutrients can impact the production or yield of secondary metabolites (Bills *et al*., 2008) or can

Table 4.3: Biological activity from Round 1 isolate fermentation extracts. Activities are calculated at 10X concentrates. Numbers represent percent inhibition. Red: bioactivity ≥80%; yellow: 60 – 80%; grey: activity not reproducible upon independently re-testing the same extract.

Bacteria	Taxonomy	Fermentation media	MRSA	VRE	CA[1]	PA[2]	PrV[3]
RKBH-B054	*Streptomyces* sp.	BFM3			99		
RKBH-B054	*Streptomyces* sp.	mMB	59		100		
RKBH-B054	*Streptomyces* sp.	mMB + HP-20			100		
RKBH-B054	*Streptomyces* sp.	mMB + Mev			100		
RKBH-B054	*Streptomyces* sp.	mMB + Prav	53		100		
RKBH-B054	*Streptomyces* sp.	BMS			98		
RKBH-B054	*Streptomyces* sp.	mF/2			99		
RKBH-B054	*Streptomyces* sp.	PEAq			98		
RKBH-B109	*Micrococcus* sp.	TSB					99
RKBH-B110	*Pseudomonas* sp.	TSB	100				97
RKVR-02	*Pseudoalteromonas* sp.	mMB		97			
RKVR-02	*Pseudoalteromonas* sp.	mMB+HP-20	100	100			
RKVR-02	*Pseudoalteromonas* sp.	mMB + Mev	50				
RKVR-02	*Pseudoalteromonas* sp.	mMB + Prav	74				
RKVR-02	*Pseudoalteromonas* sp.	TSB	61				
RKVR-04	*Pseudoalteromonas* sp.	TSB	54				96
RKVR-07	*Pseudoalteromonas* sp.	mMB+HP-20	100	100			
RKVR-07	*Pseudoalteromonas* sp.	mMB + Mev		100			
RKVR-08	*Alteromonas* sp.	BFM3	100	100			
RKVR-08	*Alteromonas* sp.	mF/2	100	100			
RKVR-11	*Janibacter* sp.	BFM3	100	100			
RKVR-11	*Janibacter* sp.	TrB	100	98			
RKVR-11	*Janibacter* sp.	PEAq	100	98			
RKVR-11	*Janibacter* sp.	mMB + Mev		51			
RKVR-11	*Janibacter* sp.	mF/2		61			
RKVR-19	*Listonella* sp.	mF/2	100	100			
RKVR-19	*Listonella* sp.	mMB + HP-20		62			
RKVR-20	*Pseudoalteromonas* sp.	mMB+HP-20	100	100			89
RKVR-20	*Pseudoalteromonas* sp.	mMB + Prav					60
RKVR-21	*Vibrio* sp.	mF/2	100	100			
RKVR-21	*Vibrio* sp.	PEAq	100	100			
RKVR-27	*Paracoccus* sp.	mMB+HP-20	100	99			
RKVR-27	*Paracoccus* sp.	BFM3	100	99			
RKVR-27	*Paracoccus* sp.	mMB + Mev		56			
RKVR-29	*Micrococcus* sp.	TSB					96
RKVR-29	*Micrococcus* sp.	mMB	100				
RKVR-30	*Ruegeria* sp.	mMB	100	98			
RKVR-30	*Ruegeria* sp.	TrB	100	99			
RKVR-30	*Ruegeria* sp.	mMB + Prav		99			
RKVR-34	*Pseudoalteromonas* sp.	mMB+HP-20	100	100			
RKVR-34	*Pseudoalteromonas* sp.	mMB + Mev	100	100			
RKVR-34	*Pseudoalteromonas* sp.	mMB + Prav		100			
RKVR-34	*Pseudoalteromonas* sp.	TSB	54	100			
RKVR-36	*Vibrio* sp.	mMB	99	99			
RKVR-36	*Vibrio* sp.	mMB+HP-20	100	100			
RKVR-36	*Vibrio* sp.	TSB	100	100			
RKVR-36	*Vibrio* sp.	BFM3	100	91			
RKVR-39	*Vibrio* sp.	BFM3	100	100			
RKVR-39	*Vibrio* sp.	mF/2	100	98			

[1] *Candida albicans*
[2] *Pseudomonas aeruginosa*
[3] *Proteus vulgaris*

produce substantially different compounds (Chen *et al*., 2001). It was also immediately clear from the present study that media selection was a critical factor in influencing the bacteria to produce antimicrobial extracts. Of these, the media most successful at stimulating cultures to produce antimicrobial agents was BFM3 and mMB + HP-20 (Table 4.3). The latter observation is not surprising, since precedence in the literature suggests the addition of adsorbent resins during fermentation greatly increases the likelihood of detecting or increasing the yield of secondary metabolites (Lee *et al*., 2003). Specifically, Diaion® HP-20 resin (matrix: styrene-divinylbenzene) was selected in this investigation as it was the most effective adsorbent resin of four resins (Amberlite™ XAD-16, Diaion® HP-20, charcoal, and silica gel) examined in a previous study, yielding a 4.2 fold increase in the quantities of the natural product teicoplanin (Lee *et al*., 2003). For example, when added at a 5% concentration during fermentation, Diaion® HP-20 prevented lethal effects on bacterial growth and reduced feedback inhibition of teicoplanin (Lee *et al*., 2003). This example highlights the ability of adsorbent resins to sequester natural products produced during fermentation, which likely prevent their detection and subsequent degradation by the microorganism. However, the most versatile bacterium in the present study was *Streptomyces* sp. RKBH-B54, which demonstrated potent bioactivity against *C. albicans* when fermented in eight of the ten fermentation media tested.

Conversely, the culture media which was the least successful at stimulating the production of antimicrobial metabolites production was TrB and BMS (Table 4.3). The lack of efficacy in the case of terrestrial broth (TrB), a culture medium devoid of salt, suggests that salts may be critical for natural product production. This can be shown in the example that no antifungal activity could be observed when this bacterium was fermented in TrB and TSB. The inability of RKBH-B54 to produce the antifungal agent when fermented in TrB, as was

characteristic for the majority of bacteria in this investigation, strongly suggests this isolate is dependent on salts for the production of secondary metabolites. However, although bacterial natural product production is strongly subject to preferred fermentation conditions (*e.g.* orbital shaking, nutrient composition, addition of adsorbent resins), the fact remains that very little is known regarding bacterial nutrition to promoting secondary metabolite production, and therefore a wide variety of conditions and/or approaches are often used as a means to compensate for our limited understanding. Taken together, however, it is suggested that future microbial fermentations from marine bacteria should incorporate HP-20 resins in a larger selection of fermentation media in addition to salts to increase the biological activity of cultures.

Confirming antimicrobial reproducibility in subsequent fermentations

Observations from antimicrobial screening from the first round of fermentation extracts were used to guide the selection of isolates and to select the most appropriate media at stimulating the production of antimicrobials in subsequent fermentations. Of the 15 isolates selected for re-fermentation, only five ACN extracts were active at 125 µg/mL (Table 4.4). This included the Gram negative bacteria *Pseudoalteromonas* spp. (RKVR-02, 07, 20, and 34) and a Gram positive bacterium *Streptomyces* sp. (RKBH-B54). No coral-associated bacterial extracts were reproducibly active against the Gram negative pathogen *P. vulgaris*. Specifically, ACN extracts from the genetically related *Pseudoalteromonas* spp. (RKVR-02, RKVR-07, and RKVR-34, 0.34 – 0.52 mg of yellow-pigmented solids) were reproducibly active against MRSA and VRE when fermented and extracted from mMB + HP-20 (Table 4.4). ACN extracts (0.21 mg) from *Pseudoalteromonas* sp. RKVR-20 were moderately active (~60% inhibition) against MRSA when grown in seed media (MB) under static conditions, yet no activity was observed

Table 4.4: Reproducible biological activity from fermentation extracts. All extract bioactivities were calculated at 125 µg/mL and all bioactivities are reported from ACN extracts except RKBH-B54, which was active in the aqueous fraction. Numbers represent percent inhibition. Brackets represent extraction from isolates grown in TSB media. Replicates (A and B) represent bioactivity from fermentations performed in duplicate.

Bacteria	Taxonomy	Media Name	MRSA	VRE	CA	PA	PrV
RKBH-B54 (A)	*Streptomyces* sp.	BFM3			100		
RKBH-B54 (B)	*Streptomyces* sp.	BFM3	59		100		
RKVR-02 (A)	*Pseudoalteromonas* sp.	mMB + HP20	100	100			
RKVR-02 (B)	*Pseudoalteromonas* sp.	mMB + HP20	100	100			
RKVR-07 (A)	*Pseudoalteromonas* sp.	mMB + HP20	100	100			99 (TSB)
RKVR-07 (B)	*Pseudoalteromonas* sp.	mMB + HP20	100	100			97 (TSB)
RKVR-20	*Pseudoalteromonas* sp.	MB	60				
RKVR-34 (A)	*Pseudoalteromonas* sp.	mMB + HP20	100	100			96 (TSB)
RKVR-34 (B)	*Pseudoalteromonas* sp.	mMB + HP20	98	100			96 (TSB)

Inhibition >80%
Inhibition 50 - 79%
Activity not reproducible

when this isolate was fermented with orbital shaking in fermentation media (data not shown). Aqueous extracts from *Streptomyces* sp. RKBH-B54 were active against *C. albicans*.

It is intriguing to note that the aforementioned *Pseudoalteromonas* spp. are related genetically (99% sequence identity to *P. piscicida* (NR_040946.1), refer to Table B2), yet displayed different growth preferences and rates. For example, the yellow-pigmented and genetically related isolates *Pseudoalteromonas* spp. RKVR02, RKVR07, and RKVR34 (referred to as "RKVR02-related isolates" herein) grew quickly on solid media and remained viable for up to one week. In addition, these isolates remained viable when grown in liquid media under aerobic conditions. In contrast, the maroon-pigmented isolate RKVR20 grew quickly on agar, yet did not remain viable for longer than 24 h on solid media. Optimum growth for this isolate was observed when it was fermented under static conditions, and transfer to fermentation media from seed resulted in a cessation of growth, pigment production, and antimicrobial activity in subsequent fermentations. This observation is not surprising, as previous reports have documented the inability of *Pseudoalteromonas* sp. to produce pigments during orbital shaking (Speitling *et al*., 2007).

The lack of reproducibility in subsequent fermentations suggests that bacterial natural product production is sensitive to variations in individual fermentations. Generally, factors limiting natural product production are the high toxicity of secondary metabolites and self-inhibition of the microorganisms to their own natural product (Hua *et al*., 2007; Lee *et al*., 2003), a phenomenon known as feedback inhibition or autoinhibition. Feedback inhibition is a mechanism by which an organism controls the unnecessary production of secondary metabolites which are not utilized (Gerhart and Pardee, 1962). Consequently, the addition of adsorbent resins during fermentations theoretically mitigates feedback inhibition by sequestering secondary

metabolites as they are produced, ultimately preventing the producing bacterium from recognizing and degrading its own antibiotic (Lee *et al*., 2003). An alternative explanation for the lack of observed biological activity from *P. elisabethae*-associated bacterial extracts is that extracts may have biological activity outside of the scope of this report. For example, a previous report of the closely related Japanese stony coral-associated *Flavobacteriaceae* sp. (98% sequence identity to RKVR24) produced the major carotenoids (3R)-saproxanthin and (3R, 3'R)-zeaxanthin, the former of which is rarely found in nature yet demonstrated significant antioxidative activities against lipid peroxidation and also demonstrated protective effects from L-glutamate toxicity (Shindo *et al*., 2007). The preceeding example suggests that the biological activity of a particular extract may have been overlooked in this investigation.

Chemical profiling of *Pseudoalteromonas* spp. by UPLC/HRMS

It is important to note that the high resolution mass spectrum is used to determine the molecular formula and molecular weight of an unknown compound through the identification of molecular ion peaks (Pavia *et al*., 2001). However, in ESI-MS, "adducts" or "pseudomolecular ions" are commonly encountered instead of the molecular ion. These species are analyte complexes generally formed with alkali metals and salts, and occur because the removal of salts from the mass spectrometer prior to analysis is virtually impossible (Schug, 2002). In positive ionization mode, many of these adducts represent the addition of a proton ($[M + H]^+$) or sodium ($[M+Na]^+$) to the molecule, which form due to the presence of an acid in the solvents (Ardrey, 2003; Schug, 2002). The loss of water ($[M+H-H_2O]^+$) is also a commonly encountered molecular fragment.

UPLC/HRMS screening of ACN extracts from *Pseudoalteromonas* sp. RKVR02-related isolates (including RKVR07 and RKVR34) revealed a variety of high molecular weight $[M+H]^+$ ions were present with a *m/z* of 844.2943 (RT = 3.08 min; $\lambda_{max(MeOH)}$ = 374 nm), 860.3084 (RT = 3.24 min; $\lambda_{max(MeOH)}$ = 391 nm), 872.3093 (RT = 3.34 min; $\lambda_{max(MeOH)}$ = 391 nm) and 924.2036 (RT = 3.34 min; $\lambda_{max(MeOH)}$ =391 nm; Figure 4.1). The mixture of compounds individually was abundant based on ELSD, a universal detector which measures the amount of light scattered by evaporated particles of eluent. Proton adducts could be confidently assigned as a result of detecting the corresponding $[M+Na]^+$ adducts. The equal peak intensities at *m/z* 844.2943 and *m/z* 846.2922 (RT = 3.06 min) matched the M+2 profile characteristic of a molecular structure containing bromine, where isotope peak intensities of each peak were roughly 1:1 (Pavia *et al*., 2001). Based on a database search for this compound, a molecular formula of $C_{38}H_{50}BrN_7O_{10}$ was proposed, which matched the structure for bromoalterochromide A (**1**, Figure 4.2). Bromoalterochromide A (λ_{max} = 395 nm) was isolated from an Australian sponge-associated *Alteromonas* sp. and demonstrated cytotoxic activity against sea urchin (*Strongylocentrotus intermedius*) eggs with an MIC of 40 µg/mL (Speitling *et al*., 2007). However, no antibiotic activity was observed from this compound. Considering the taxonomic confusion regarding *Pseudoalteromonas* and *Alteromonas* in the literature (Bowman, 2007) in addition to the metabolite production from another marine invertebrate, there is a high probability that *P. elisabethae*-associated *Pseudoalteromonas* sp. RKVR02-related isolates are also a source of Bromoalterochromide A.

Although Bromoalterochromide A is the likely identity of the metabolite produced by *Pseudoalteromonas* sp. RKVR02 and related isolates, the three remaining compounds (*m/z* 860.3084 $[M+H]^+$, 872.3093 $[M+H]^+$, and 924.2036 $[M+H]^+$) may be novel based on a lack of

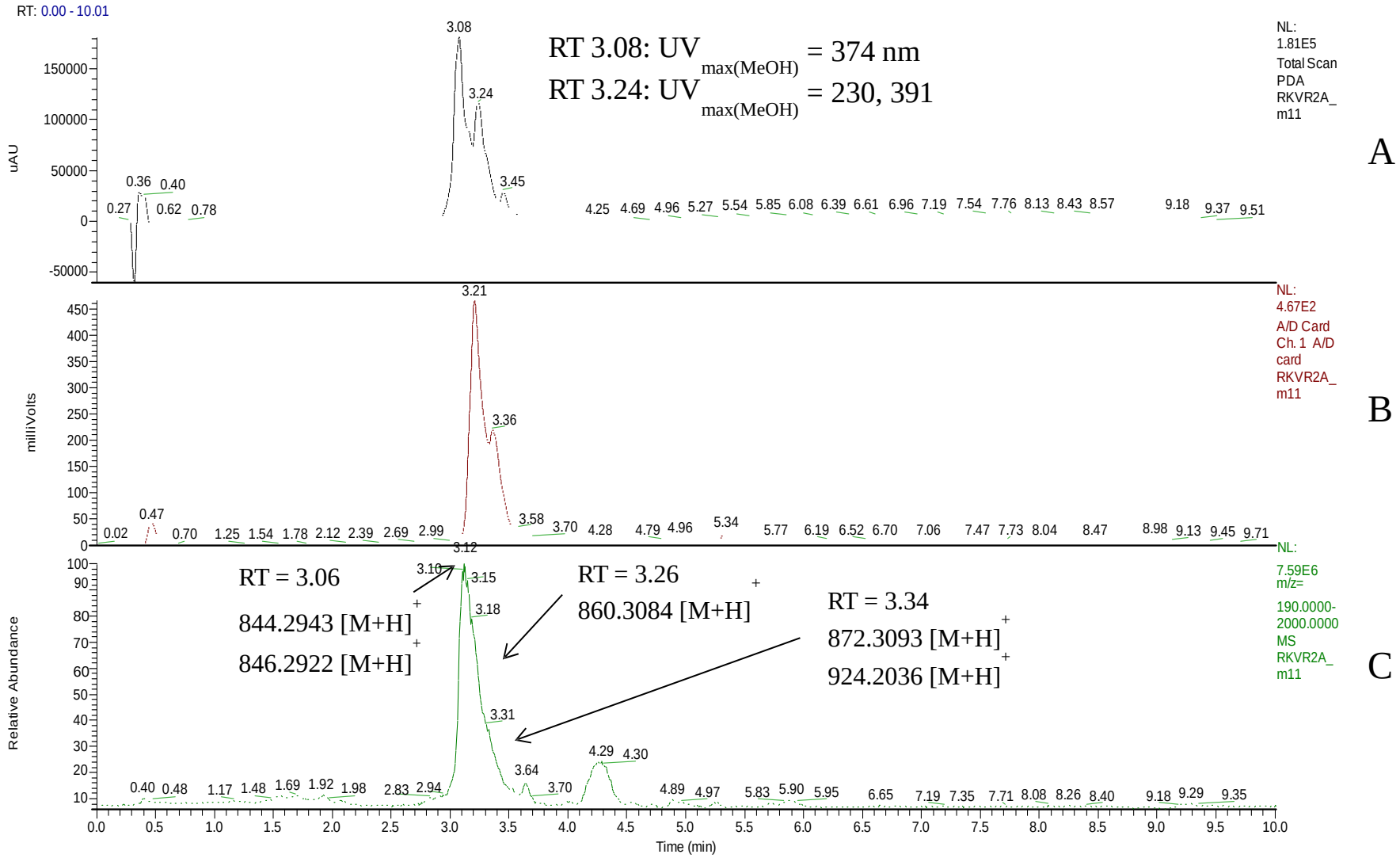
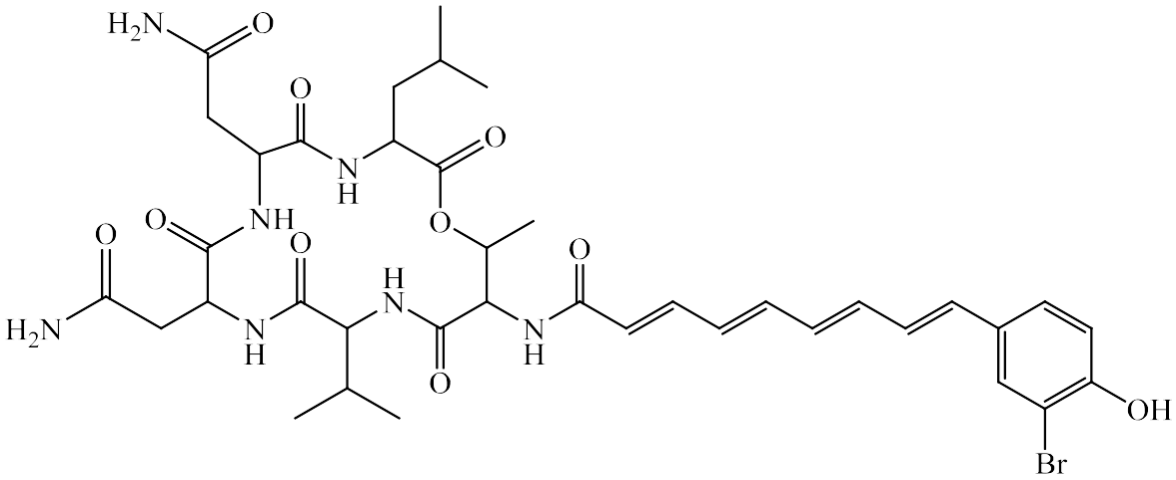


Figure 4.1: UPLC/HRMS profile from *Pseudoalteromonas* sp. RKVR02. A variety of high molecular weight $[M+H]^+$ pseudomolecular ions are shown. A) UV; B) ELSD; C) MS demonstrating the presence of high molecular weight compounds.



Bromoalterochromide A (**1**)
Chemical Formula: $C_{38}H_{50}BrN_7O_{10}$
Exact Mass: 843.28025

Figure 4.2: The chemical structure for Bromoalterochromide A, a likely metabolite isolated from *Pseudoalteromonas* spp. RKVR02, RKVR07, and RKVR34.

plausible identities when searching nominal masses for protonated ions. For example, only four database matches were identified for *m/z* 860.3084 ($[M+H]^+$; RT = 3.24 min) and 872.3093 ($[M+H]^+$; RT = 3.34 min) respectively. Similarly, searching the nominal mass for *m/z* 924 (RT = 3.34 min) generated six hits. However, none of the database matches were likely candidates, as none of the published accurate masses were within the accepted 5 ppm range to assign a putative structure or molecular formula (Gross, 1994). Consequently, these metabolites are currently a subject of biological investigation and structural characterization within the Kerr lab.

In contrast to the more complex spectrum observed for RKVR02-related isolates, the chemical profile of RKVR20 was less complex when analyzed in positive ionization mode (Figure 4.3). For example, a pseudomolecular ion with m/z 817.5867 $[M+H]^+$ was most abundant in RKVR20 based on ELSD data (Figure 4.3). This corresponds to peaks at RT 3.42 and 3.62 minutes ($\lambda_{max(MeOH)}$ = 533 nm, Figure 4.3). However, no hits were generated from Antibase 2007 by searching +-ESI-H^+ for 817.6. Furthermore, only five hits were generated when searching the nominal mass, yet none were within the acceptable chemical shift range for HRMS (≤5 ppm; Gross, 1994). In this case, the compound did not appear to be brominated based on a lack of equal intensity peaks in the high resolution mass spectrum. Bioassay guided fractionation and NMR structural elucidation is required to purify and determine the structure of these metabolites. This is also a subject of future investigation within the Kerr lab.

Pseudoalteromonas spp. as a recently described source of natural products

A review of the literature regarding natural products from marine bacteria suggests that members from the recently described taxonomic group *Pseudoalteromonas* sp. are an exceptional resource of secondary metabolites (Bowman, 2007; Shnit-Orland *et al*., 2012) which have only recently begun to be investigated. These members are commonly found in

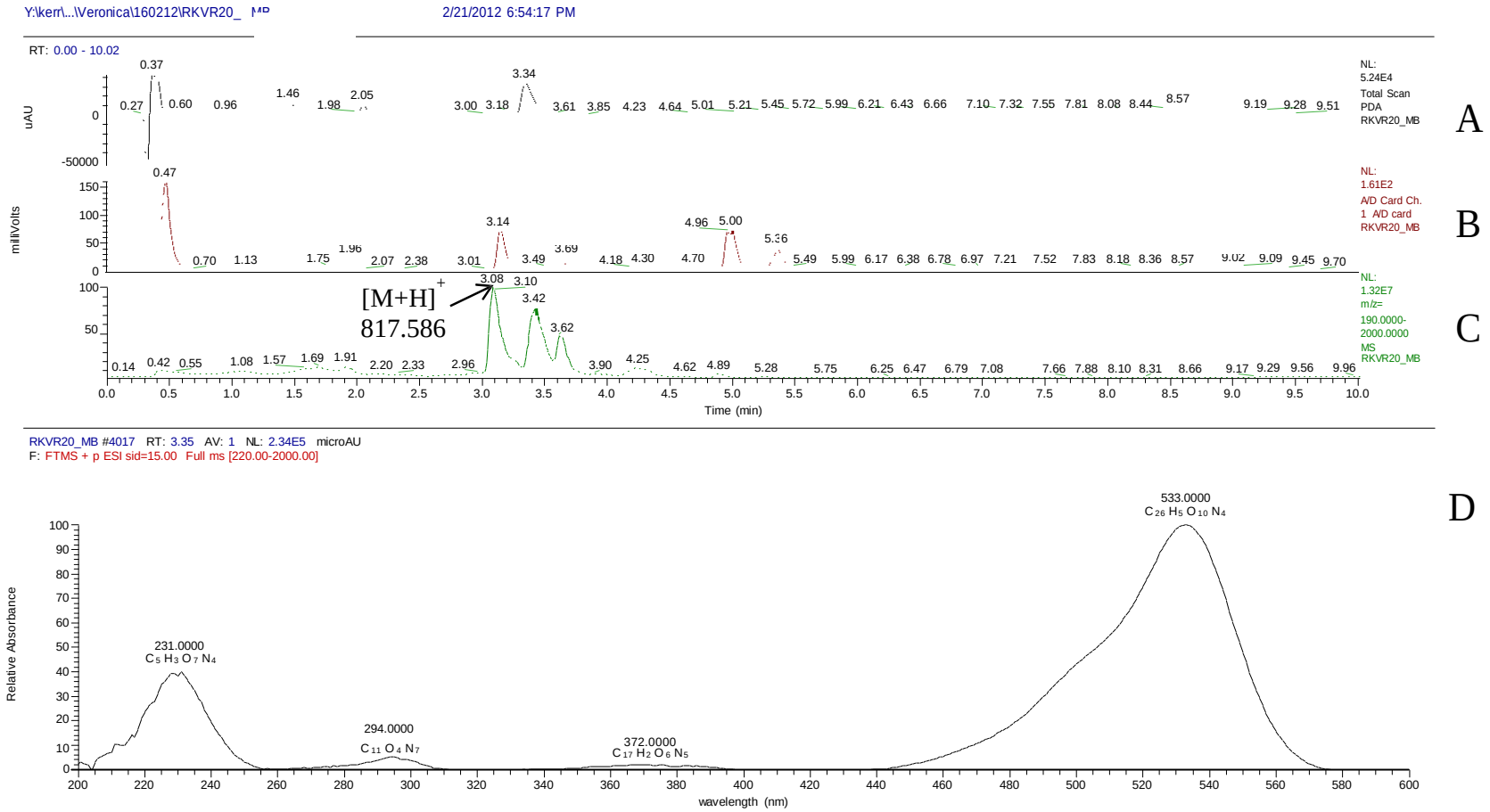


Figure 4.3: UPLC/HRMS profile from *Pseudoalteromonas* sp. RKVR20. A) UV; B) ELSD; C) MS; D) UV trace demonstrating a $UV_{max(MeOH)}$ of 231 and 533 nm at RT = 3.34 min. The $[M+H]^+$ pseudomolecular ion at RT = 3.08 min is highlighted on the high resolution mass spectrum (C).

association with higher marine organisms and appear to play potentially symbiotic roles (Franks *et al*., 2005). For example, *Pseudoalteromonas* spp. isolated from Red Sea stony corals demonstrated extracellular Gram positive bioactivity, yet were inactive against Gram negative bacteria (Shnit-Orland *et al*., 2012). Specifically, low molecular weight compounds with antimicrobial activity have been published (Bowman, 2007; Isnansetyo and Kamei, 2003). For example, literature reports of low molecular weight compounds from *Pseudoalteromonas* sp. are active against MRSA and *E. faecalis* (MIC = 1 – 2 µg/mL) without any cytotoxic effects (Isnansetyo and Kamei, 2003). In contrast, less information is known regarding high molecular weight substances such as those described in the present study. However, a dissertation reported brominated high molecular weight compounds ranging from 830 – 954 Da were produced by *P. piscicida* and demonstrated broad spectrum antimicrobial activity, although their structures were unidentified (Bowman, 2007).

The complexity of the chemical profiles from *Pseudoalteromonas* sp. RKVR02-related isolates in addition to the weak antibacterial activity and the difficulties in maintaining viable cultures of RKVR20 made *Pseudoalteromonas* spp. poor candidates for bioassay guided fractionation. Conversely, the strong and reproducible antifungal activity from *Streptomyces* sp. RKBH-B54 extracts made this isolate a prime candidate for further purification via bioassay guided fractionation. Consequently, efforts were focused on the identification of novel antifungal compounds or novel analogues from *Streptomyces* sp. RKBH-B54.

Bioassay guided fractionation of *Streptomyces* sp. RKBH-B54

Crude aqueous extracts from *Streptomyces* sp. RKBH-B54 demonstrated remarkably potent and reproducible antifungal activity against *C. albicans*, and for this reason the extract

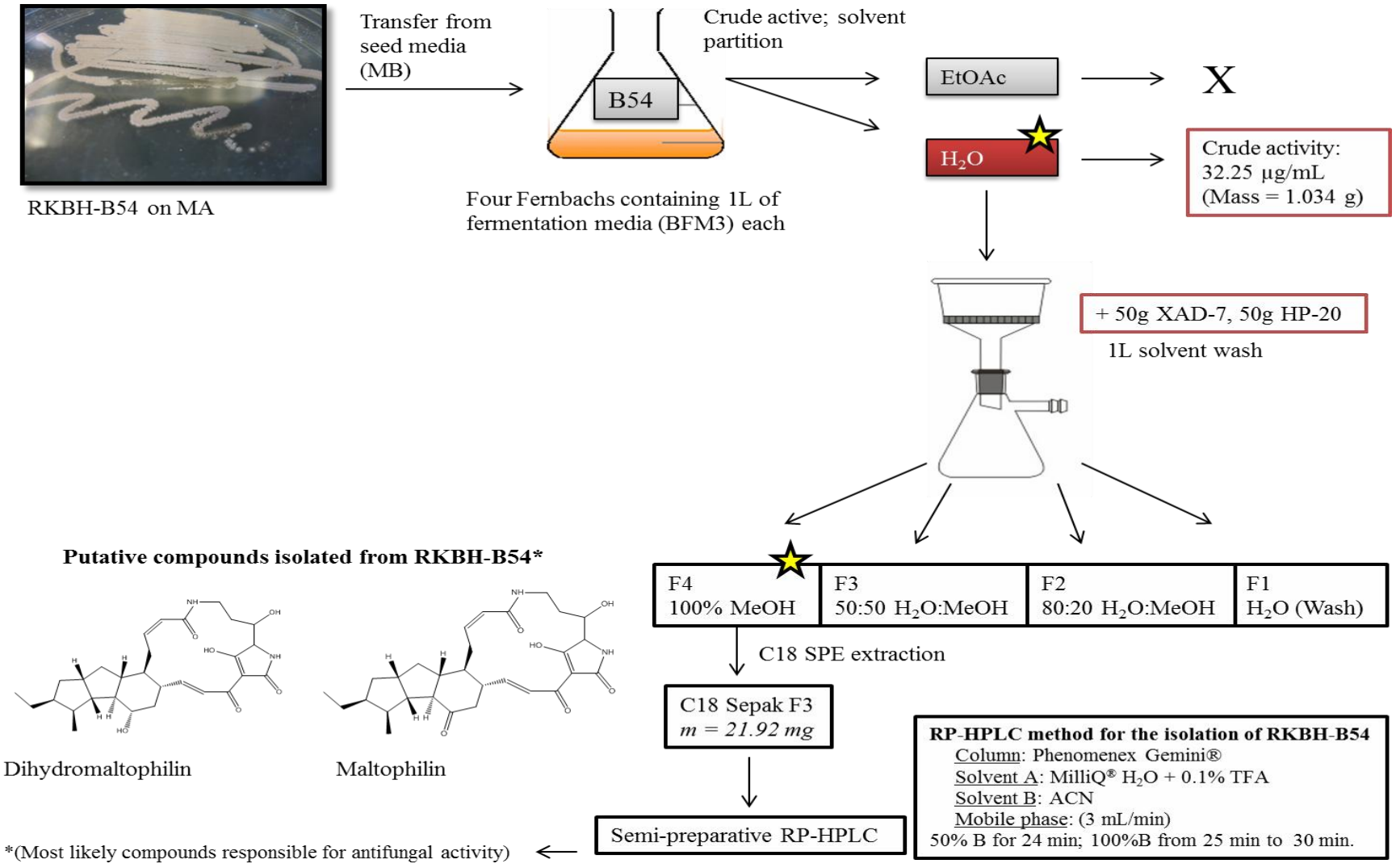
was purified further. To accomplish this, large scale (4L) fermentation and extraction was used to generate increased amounts of the antifungal metabolite(s). Large scale fermentations are necessary in microbial natural product isolation and structural elucidation efforts as a consequence of the often low yields of the compound of interest amongst a variety of additional bacterial metabolites and media components (Berrué *et al.*, 2011). Furthermore, in this study, the combined small scale fermentation (200 mL) extract was less bioactive than the large scale fermentation extract (Table 4.5); therefore, bioassay guided purification was performed on the large scale fermentation extract only as summarized in Scheme 4.1.

Following large scale fermentation, metabolites were extracted with 5% each of activated Diaion® HP-20 and Amberlite® XAD-7. These resins were selected to ensure that salts and polar compounds could be removed from the aqueous fraction by washing the resins with H_2O, yet would adsorb less polar metabolites which could be easily eluted using common organic solvents (Ebada *et al*., 2008). Next, partitioning the extract with equivalent volumes of aqueous and organic solvents resulted in a crude brown aqueous extract (1.97 g) which was capable of strongly and reproducibly inhibiting *C. albicans* (crude bioactivity IC_{50} = 32.3 µg/mL). The presence of bioactivity in the water layer (and absence of activity in media control) suggests that the antifungal compound(s) contained polar groups (Buckingham, 1993). In contrast, the organic fractions (EtOAc, ACN, and hexane) were not biologically active at 125 µg/mL (Table 4.5). A major concern in purifying metabolites from aqueous extracts, particularly from marine bacteria, is the co-extraction of a high concentration of salt present within the fermentation media, as salts can interfere with downstream purification (Vizcaino, 2011). Specifically, the fermentation medium BFM3 contains 18g/L Instant Ocean as a salt source, and as salts are soluble in H_2O

Table 4.5: Bioactivities of RKBH-B54 extracts. “ls” refers to biological activities from large scale fermentations and “ss” refers to biological activities from small scale fermentations. Numbers represent percent inhibition. However, *C. albicans* grows in clumps and although some values appear to be within the "hit" range, visible growth was observed in the microtiter plate. Consequently, these values resemble media blanks and therefore represent false positives.

	B54 Percent inhibition (µg/mL)						
Fraction	250	125	62.5	31.3	15.6	7.8	3.9
B54 ls H_2O	99	99	99	96	12	6	0
B54 ls H_2O	100	99	99	96	2	1	-5
B54 ls EtOAc	95	95	98	94	58	36	4
B54: ls ACN	57	68	74	84	92	66	71
B54: ss H_2O	99	70	-10	-1	8	-5	-7
B54 ss EtOAc	90	29	47	30	52	34	-3
B54 ss ACN	76	62	28	45	52		
Blank	100	100	100				
Organism blank	3	0	-3				
Nystatin % inhibition (dilution series; µg/mL)	6.4	3.2	1.6	0.8	0.4	0.2	
Nystatin	100	100	100	35	2	7	

≥80 inhibition
C. albicans + samples
Media blank
C. albicans blank
C. albicans + Nystatin
False positive



Scheme 4.1: A summary of the workflow of the bioassay-guided fractionation employed for *Streptomyces* sp. RKBH-B54.

(*e.g*. 35.85 g NaCl per 100 g H_2O at 25°C), the aqueous nature of the extract suggested salt removal was necessary prior to chromatographic separation. Consequently, a protocol was designed to remove salts from the extract by washing resins containing the adsorbed metabolites using increasing concentrations of MeOH in H_2O (refer to Materials and Methods). Biological activity screening of each solvent wash revealed F4 (100% MeOH) was the most active fraction against *C. albicans* with a minimum inhibitory concentration (MIC) of 0.6 µg/mL; a value comparable to the positive control Nystatin (Table 4.6).

Chemical analysis of *Streptomyces* sp. RKBH-B54 by UPLC/HRMS

Next, the chemical profile of fraction F4 was analyzed to assign a putative molecular formula for the antifungal compound(s). Media blanks and MeOH blanks were also analyzed and compared as negative controls to ensure that potential antifungal compounds were only detected in active fractions. The most abundant peaks in F4 based on ELSD (Figure 4.4B) corresponded to several pseudomolecular ions in the mass spectrum (Figure 4.4C). Fraction 4 contained eight identifiable proton adduct pseudomolecular ions with a *m/z* of 943.5304 ($[M+H]^+$, RT = 3.08 min); 511.2804 ($[M+H]^+$, RT = 3.08 min); 1109.5820 ($[M+H]^+$, RT = 3.11 min); 1111.5986 ($[M+H]^+$, RT = 3.14 min); 527.2756 ($[M+H]^+$, RT = 3.14 min); 513.2955 ($[M+H]^+$, RT = 3.40 min); 511.2805 ($[M+H]^+$, RT = 3.65 min); and 497.3005 ($[M+H]^+$, RT = 3.65 min; Figure 4.4). Confidence in the abilities to assign ions as the $[M+H]^+$ adduct was again assured based on the evidence of associated ($[M+Na]^+$ and/or ($[M+H-H_2O]^+$ adducts. UV analysis indicated F4 contained the presence of a conjugated or aromatic system, as reflected by the ability to absorb UV light at $\lambda_{max(MeOH)}$ = 280, 325 nm (RT = 3.31 and 3.54 min; Figure 4.4A). Additionally, a UV

Table 4.6: Biological activity screening of RKBH-B54 extracts against *C. albicans* from increasing MeOH concentrations. Values are recorded as percent inhibitions.

% inhibition (ug/mL)	500	250	125	62.5	31.25	15.6	7.8	3.9	2	0.98	0.49	0.24
F1 (H_2O)	94	44	-18	28	-49	-9	-16	9	3	-11	-7	-10
F2 (80:20 H_2O:MeOH)	-32	-22	-26	-7	-36	-25	1	25	7	-34	13	-24
F3 (50:50 H_2O:MeOH)	90	61	-10	17	-57	-27	-26	21	-6	-11	-13	-24
F4 (100% MeOH)	97	98	99	100	98	97	97	100	97	96	39	-7
Controls	100	100	100	-10	8	2	99	98	99	89	12	-19
						Nystatin (µg/mL)	8	4	2	1	0.5	0.25

C. albicans with sample
≥ 80% inhibition
Inhibition 50 - 79%
Blank
C. albicans blank
C. albicans with Nystatin

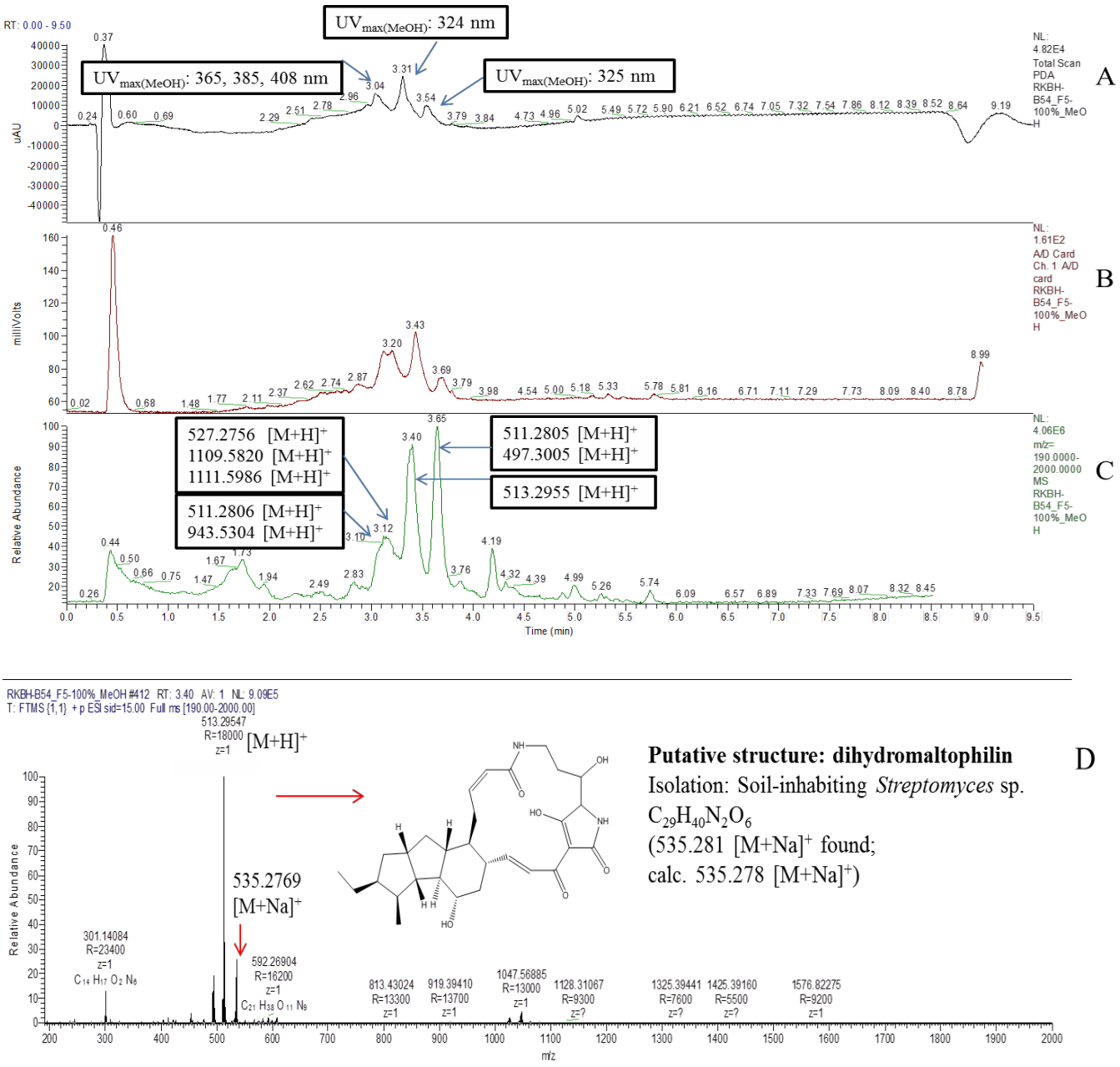


Figure 4.4: UPLC/HRMS profile of 100% MeOH (F4) from RKBH-B54 showing identified $[M+H]^+$ pseudomolecular ions. A) UV trace showing $\lambda_{max(MeOH)}$ for each UV peak; B) ELSD trace showing relative abundances; C) MS highlighting identified $[M+H]^+$ pseudomolecular ions, and D) MS at RT 3.40 min showing the putative structure for the compound with *m/z* 513.2955.

spectrum matching the polyene group of antibiotics was identified with a $\lambda_{max(MeOH)}$ = 365, 385, and 408 nm (RT = 3.04 min, Figure 4.4; Jain and Jain, 2007; Seipke *et al*., 2011). No UV absorption or ELSD peaks were observed in media blanks and MeOH blanks, suggesting the above-mentioned compounds were the product of bacterial fermentations.

Identification of metabolites from *Streptomyces* sp. RKBH-B54

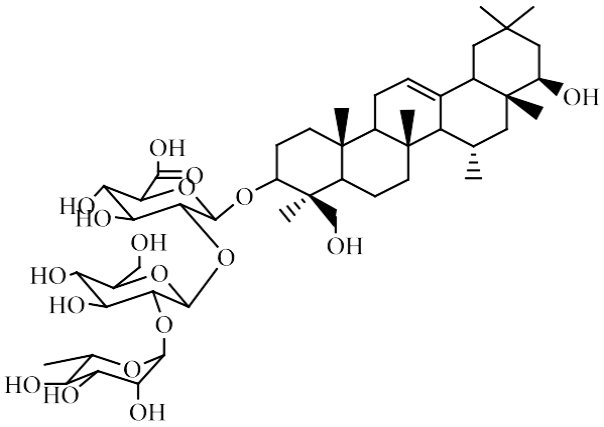
At this stage, a database search was conducted to propose molecular formulae and potential molecular structures for the compounds isolated from F4 (summarized in Figure 4.5 and Table 4.7). Each $[M+H]^+$ pseudomolecular ion was compared to published compounds by searching positive mode proton or sodium adducts in Antibase 2007.

***m/z* 943**

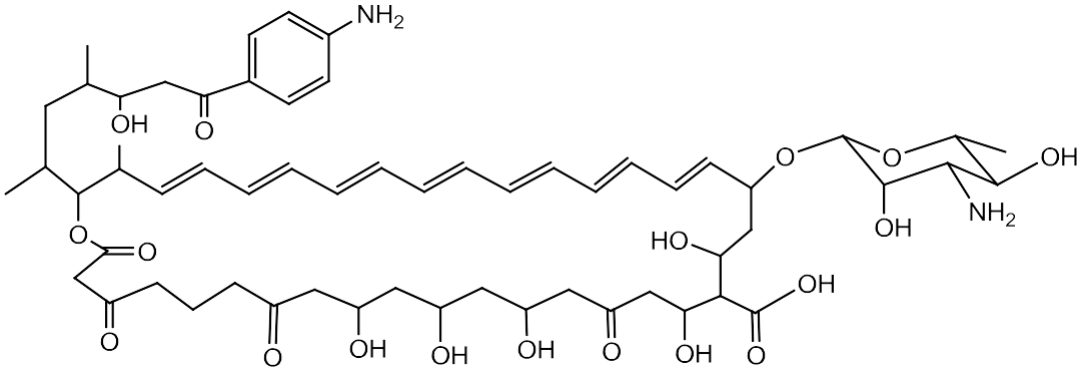
Thirteen database matches were recorded from searching for the nominal mass for the $[M+H]^+$ pseudomolecular ion at *m/z* 943.5304 (RT = 3.08 min), yet the closest database match was the fungal-derived compound Soyasaponine I (**2**; Figure 4.5), with a molecular formula of $C_{49}H_{78}O_{18}$ (calculated $[M+H]^+$ 943.5268; δ = 3.8 ppm; Maul *et al*., 1999). However, it is rare for bacteria and fungi to share similar secondary metabolic pathways (Blodgett *et al*., 2010) and thus it is likely that the ion with *m/z* 943 represents a novel compound.

***m/z* 1109/1111**

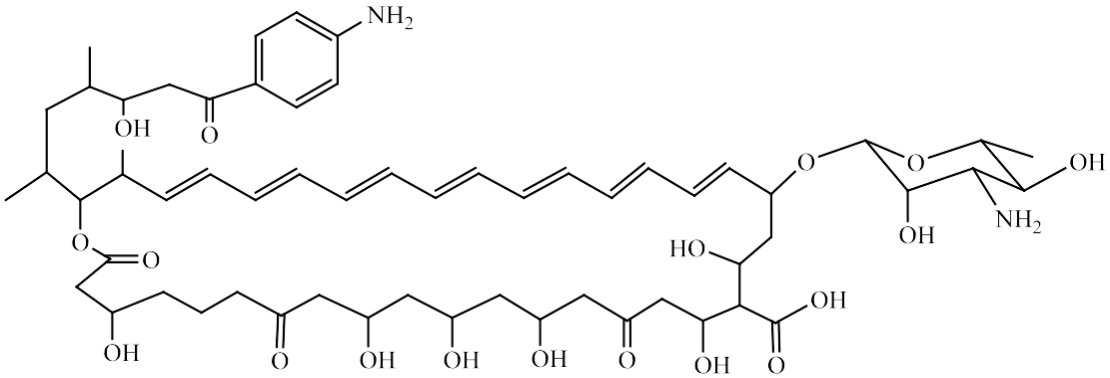
The pseudomolecular ions at $[M+H]^+$ 1109/1111 (RT = 3.08 min) were proposed as candicidin (Levorin) analogues A2 (**3**) and A3 (**4**; Figure 4.5), with putative molecular formulae of $C_{59}H_{48}N_2O_{18}$ and $C_{59}H_{46}N_2O_{18}$ respectively (δ = 3 ppm). Incidentally, candicidin is an



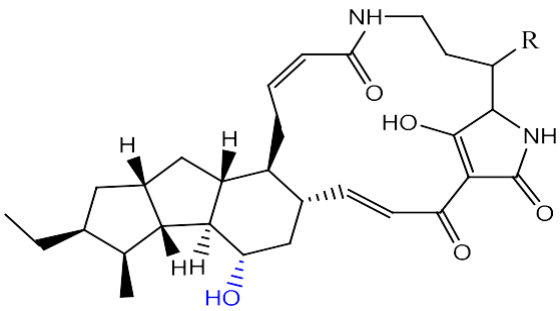
Soyasaponine I **(2)**
Chemical Formula: $C_{48}H_{78}O_{18}$
Exact Mass: 942.51882



Levorin-A2; Candicidin D **(3)**
Chemical Formula: $C_{59}H_{84}N_2O_{18}$
Exact Mass: 1108.57191

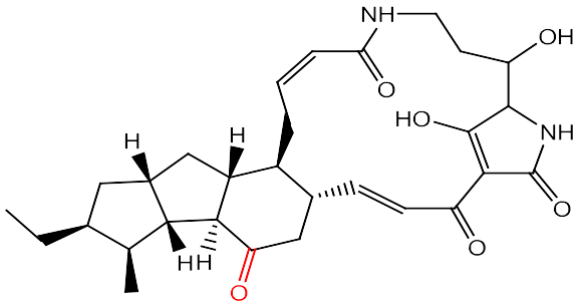


Levorin-A3 **(4)**
Chemical Formula: $C_{59}H_{86}N_2O_{18}$
Exact Mass: 1110.58756

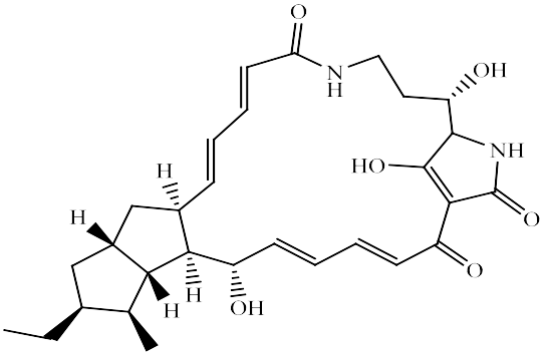


*Dihydromaltophilin (**5**): R=OH
Chemical Formula: $C_{29}H_{40}N_2O_6$
Exact Mass: 512.28864

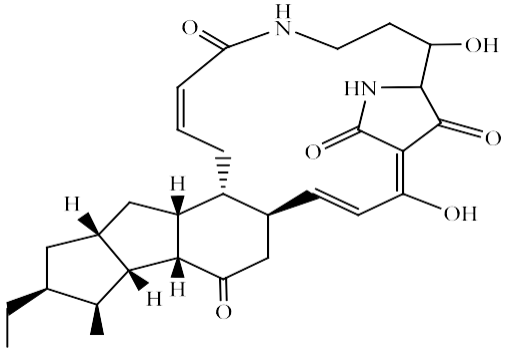
3-deOH HSAF (**9**): R=H
Chemical Formula: $C_{29}H_{40}N_2O_5$
Exact Mass: 496.29372



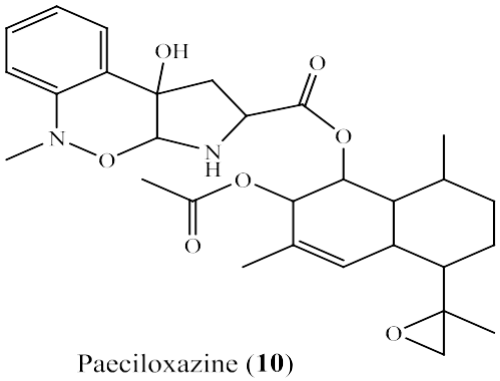
*Maltophilin (**6**)
Chemical Formula: $C_{29}H_{38}N_2O_6$
Exact Mass: 510.27299



Alteramide A (**7**)
Chemical Formula: $C_{29}H_{38}N_2O_6$
Exact Mass: 510.27299



Xanthobaccatin-A (**8**)
Chemical Formula: $C_{29}H_{38}N_2O_6$
Exact Mass: 510.27299



Paeciloxazine (**10**)
Chemical Formula: $C_{29}H_{38}N_2O_7$
Exact Mass: 526.26790

Figure 4.5: Proposed compounds isolated from RKBH-B54. *Proposed compounds causing antifungal activity from RKBH-B54.

Table 4.7: Summary of pseudomolecular ions detected in *Streptomyces* sp. RKBH-B54 fraction 4. [F]; fungi; [B]; bacteria.

Structure Number	$[M+H]^+$	$[M+Na]^+$	$[M+H-H_2O]^+$	RT (min)	Putative Molecular Formula	Plausible Compound	Source	Bioactivity	Reference
2	943.5304	965.5118	-	3.08	$C_{49}H_{78}O_{18}$	Soyasaponine I	[F] GT 41117	None reported	Maul *et al*., 1999
3	1109.582	1131.318	1091.5719	3.11	$C_{59}H_{48}N_2O_{18}$	Candicidin; Levorin-A2	[B] *Streptomyces griseus, S. levoris*	Antifungal; antibacterial; antiviral	Kozhuharova *et al*., 2008; Xiang-zhao *et al*., 2005
4	1111.5986	1133.581	1093.5876	3.14	$C_{59}H_{46}N_2O_{18}$	Levorin-A3	[B] *Streptomyces levoris, S. helvoloviolaceus*	Antifungal; antibacterial; antiviral	Kozhuharova *et al*., 2008; Xiang-zhao *et al*., 2005
5	513.2955	535.2769	-	3.40	$C_{29}H_{40}N_2O_6$	Dihydromaltophilin	[B] *Streptomyces* sp.; *Lysobacter enzymogenes*	Antifungal	Graupner *et al*., 1997
6	511.2806	533.2625	493.2701	3.65	$C_{29}H_{38}N_2O_6$	Maltophilin	[B] *Stenotrophomonas* sp.	Antifungal	Jakobi *et al*., 1996
7	511.2806	533.2625	493.2701	3.65	$C_{29}H_{38}N_2O_6$	Alteramide A	[B] *Alteromonas* sp. collected from the sponge *Halichondria okadai*	Murine leukemia and lymphoma	Shigemori *et al*., 1992
8	511.2806	533.2625	493.2701	3.65	$C_{29}H_{38}N_2O_6$	Xanthobaccatin A	[B] *Stenotrophomonas* sp. strain SB-K88	Antifungal	Hashidoko *et al*., 1999
9	497.3005	519.2817	-	3.65	$C_{29}H_{40}N_2O_5$	3-deOH-HSAF	[B] *Lysobacter enzymogenes*	Inactive against fungi	Li *et al*., 2012
10	527.2756	549.2575	509.2648	3.14	$C_{29}H_{38}N_2O_7$	Paeciloxazine	[F] *Paecilomyces* sp.	Nematicidal	Kanai *et al*., 2004

antifungal metabolite isolated from a *Streptomyces* sp. from higher Attine ants (Seipke *et al*., 2011), and therefore was proposed as a likely metabolite produced by *Streptomyces* sp. RKBH-B54. Furthermore, the UV spectrum of F4 at RT = 3.08 min ($\lambda_{max(MeOH)}$ = 365, 385, 408 nm) displayed a UV spectrum matching the polyene class of metabolites, including the UV profile of candicidin ($\lambda_{max(MeOH)}$ = 360, 380, 403 nm; Seipke *et al*., 2011).

***m/z* 511/513**

The molecular ion at *m/z* 513.2955 $[M+H]^+$ (RT = 3.40 min) was a match to dihydromaltophilin (**5**; Figure 4.5), an antifungal compound with the molecular formula $C_{29}H_{40}N_2O_6$ (experimental $[M+Na]^+$ 535.281; calc. 535.2784; δ = -1.3 ppm) from a soil-inhabiting *Streptomyces* sp. (Graupner *et al*., 1997). The UV profile for dihydromaltophilin ($\lambda_{max(MeOH)}$ = 318 nm) shared absorption characteristics of the compound with the molecular ion at *m/z* 513.2959 ($\lambda_{max(MeOH)}$ = 324 nm). A compound with a *m/z* 511.2804 eluted before (RT = 3.08 min) and after (RT = 3.65 min) the compound at *m/z* 513, suggesting two compounds with identical molecular formulae were present, yet with different polarities. Consequently, three plausible compounds with a chemical formula of $C_{29}H_{38}N_2O_6$ (calculated $[M+H]^+$ 511.2809; δ = -1.1 ppm) were proposed: maltophilin (**6**), alteramide A (**7**), and xanthobaccin A (**8**; Figure 4.5). Based on an inspection of the proposed structures, alteramide A ($\lambda_{max(MeOH)}$ = 347 nm) is more polar than maltophilin ($\lambda_{max(MeOH)}$ = 320 nm) and xanthobaccin-A ($\lambda_{max(MeOH)}$ = 321 nm), although it is difficult to determine whether this compound is more polar than dihydromaltophilin. Alteramide A was produced by a Japanese sponge-associated *Alteromonas* sp., yet no antifungal activity was reported (HR-FAB-MS *m/z* 533.2629 $[M+Na]^+$; Shigemori *et al*., 1992). Xanthobaccin A ($\lambda_{max(MeOH)}$ = 239, 321 nm), isolated from broth cultures of

Stenotrophomonas sp. from sugar beet roots, also had antifungal activity against *Pythium ultimum* at an MIC of 1 µg/mL (Hashidoko *et al*., 1999). Maltophilin, produced by the bacterium *Stenotrophomonas maltophilia* from the rhizophere of the plant *Brassica napus* ($\lambda_{max(MeOH)}$ = 320 nm, *m/z* 511.28120 $[M+H]^+$; Jakobi *et al*., 1996) was the most likely compound with *m/z* 511 eluting at RT 3.65 min ($\lambda_{max(MeOH)}$ = 325 nm). Maltophilin is also an antifungal agent active against a variety of fungi, including *C. albicans* (Jakobi *et al*., 1996). Structurally, dihydromaltophilin and maltophilin have a similar carbon skeleton, yet differ in that maltophilin contains a ketone moiety while dihydromaltophilin contains a hydroxyl group at position C-14 (highlighted by red and blue respectively in Figure 4.5).

***m/z* 497**

The compound with *m/z* at 497.3009 was a structural match to 3-deOH-heat-stable antifungal factor (HSAF; experimental *m/z* 497.3008 $[M+H]^+$) (**9**) with a molecular formula of $C_{29}H_{41}N_2O_5$ (δ = 0.2 ppm; Li *et al*., 2012). It is worthwhile mentioning that this metabolite, which lacks the hydroxyl moiety on the ornithine residue of dihydromaltophilin, is inactive against fungi (Li *et al*., 2012).

***m/z* 527**

The nearest database match to the $[M+H]^+$ pseudomolecular ion at *m/z* 527.2756 (RT = 3.14 min) was paeciloxazine (**10**), a nematicidal compound isolated from the fungi *Paecilomyces* sp. (δ = -0.5 ppm; Kanai *et al*., 2004). This compound was the only match in the database which was within the accepted 5 ppm range, yet since these compounds are not biosynthetically related,

it is unlikely that this metabolite was produced by *Streptomyces* sp. RKBH-B54 and therefore may be a novel compound.

Purification of compounds causing antifungal activity in RKBH-B54

Consequently, the next logical step in the purification process was to target which metabolite(s) may be the most likely antifungal agent produced by *Streptomyces* sp. RKBH-B54. This was required to determine if the metabolite of interest was present in sufficient quantities to enable further purification. Based on database matches and a review of the literature, the compounds proposed to be responsible for the antifungal activity from RKBH-B54 are dihydromaltophilin (*m/z* 513, RT = 3.40 min) and/or maltophilin (*m/z* 511, RT = 3.65 min; Figure 4.5; Graupner *et al*., 1997; Jakobi *et al*., 1996). Evidently, these compounds were also the most abundant metabolites in F4 based on ELSD. Owing to the antifungal activity reported for both compounds (Graupner *et al*., 1997; Jakobi *et al*., 1996), it is possible that both compounds may be acting against *C. albicans* in a synergetic fashion. The suggestion that dihydromaltophilin and maltophilin were both produced by *Streptomyces* sp. RKBH-B54 is further supported by the report which also reports that a soil-isolated *Streptomyces* sp. is capable of producing both dihydromaltophilin and maltophilin simultaneously (Graupner *et al*., 1997).

A wealth of literature also reports the production of candicidin (the proposed compound for $[M+H]^+$ ions at *m/z* 1109 and 1111), an antifungal metabolite produced by *Streptomyces* sp., (Haeder *et al*., 2009; Seipke *et al*., 2011) and therefore may also be contributing to the observed antifungal activity from *Streptomyces* sp. RKBH-B54 at least in part. However, this compound was not pursued further because of the complexity of the chromatographic profile at RT = 3.08 min in addition to the lower quantities as indicated by ELSD. Although the antifungal

compound(s) from RKBH-B54 may have already been published, it is virtually impossible to determine the true identity of the compound without structural elucidation via NMR. However, the strong and reproducible bioactivity of the metabolite(s) in crude aqueous extracts made it an excellent target for downstream analysis, for example, to provide a standard for precursor feeding experiments. Therefore, it was rationalized that the purification of the antifungal compound(s) from RKBH-B54 is a worthwhile pursuit.

The next step in the purification process was to purify the antifungal compound(s) from F4. Prior to HPLC separation, a C18 SPE fractionation was performed since salts were originally suspected to be present based on the high ELSD signal in the solvent front of this fraction (Figure 4.4B). Biological activity screening of the C18 SPE fractions revealed F3 (21.92 mg) was at least four times more active than other fractions (Table 4.8). Although the pseudomolecular ion for the plausible antifungal metabolite candicidin ($[M+H]^+$ = 1109/1111) could no longer be detected at this stage based on the absence of the characteristic three-peak UV profile, the presence of the plausible antifungal metabolites at *m/z* 511 and 513 could be detected (Figure 4.6A – C).

An aliquot of the crude extract ("Trial 1") was chromatographed on a C18 column by RP-HPLC. In total, six fractions were generated: F1: 0.87 mg; F2: 2.21 mg; F3: 8.57 mg, yellow powder; F5: 1.19 mg; HPLC Tail: 1.11 mg (Figure 4.7). Following the first round of RP-HPLC purification in Trial 1, UPLC/HRMS analysis confirmed that HPLC F3 contained the compounds with *m/z* 513 (RT = 3.40 min) and *m/z* 511 (RT = 3.65 min; Figure 4.8) which eluted close in retention time as can be seen in the ELSD chromatogram. At this stage, the remainder of the C18 SPE fraction F3 was purified in HPLC Trial 2 to increase the amount of material for purification.

Table 4.8: Biological activity of C18 Sepak fractions from RKBH-B54 against *C. albicans*. Percent inhibitions are recorded in µg/mL. Dilutions from fractions 1 – 6 range from 250 to 0.2 µg/mL; 100% MeOH extract concentrations range from 250 – 7.8 µg/mL; H fraction bioactivities range from 1 g to 31.3 µg/mL.

% Inhibition												
	100% MeOH (100)	MeOH d1 (100)	MeOH d2 (100)	MeOH d3 (100)	MeOH d4 (100)	MeOH d5 (100)	B54_C18 H_2O (1g) (99)	H_2O d1 (93)	H_2O d2 (46)	H_2O d3 (17)	H_2O d4 (0)	H_2O d5 (0)
F1	100	100	100	100	99	95	38	21	8	0	0	0
F2	100	100	100	100	100	97	35	22	11	0	0	0
F3	100	100	100	100	100	100	100	97	71	17	0	0
F4	100	100	100	100	99	14	5	6	0	0	0	0
F5	89	85	17	11	3	7	15	9	6	3	0	0
F6	100	100	100	100	100	71	27	14	14	4	2	0
µg/mL	250.0	125.0	62.5	31.3	15.6	7.8	3.9	1.95	0.98	0.49	0.24	0.12
	100	100	100	-21	16	6	87	100	100	22	5	-16
						Nystatin percent inhibition	6.4	3.2	1.6	0.8	0.4	0.2

≥80% inhibition
>60-80% inhibition
Organism with Samples
Blank
Organism blank
Organism with Antibiotic

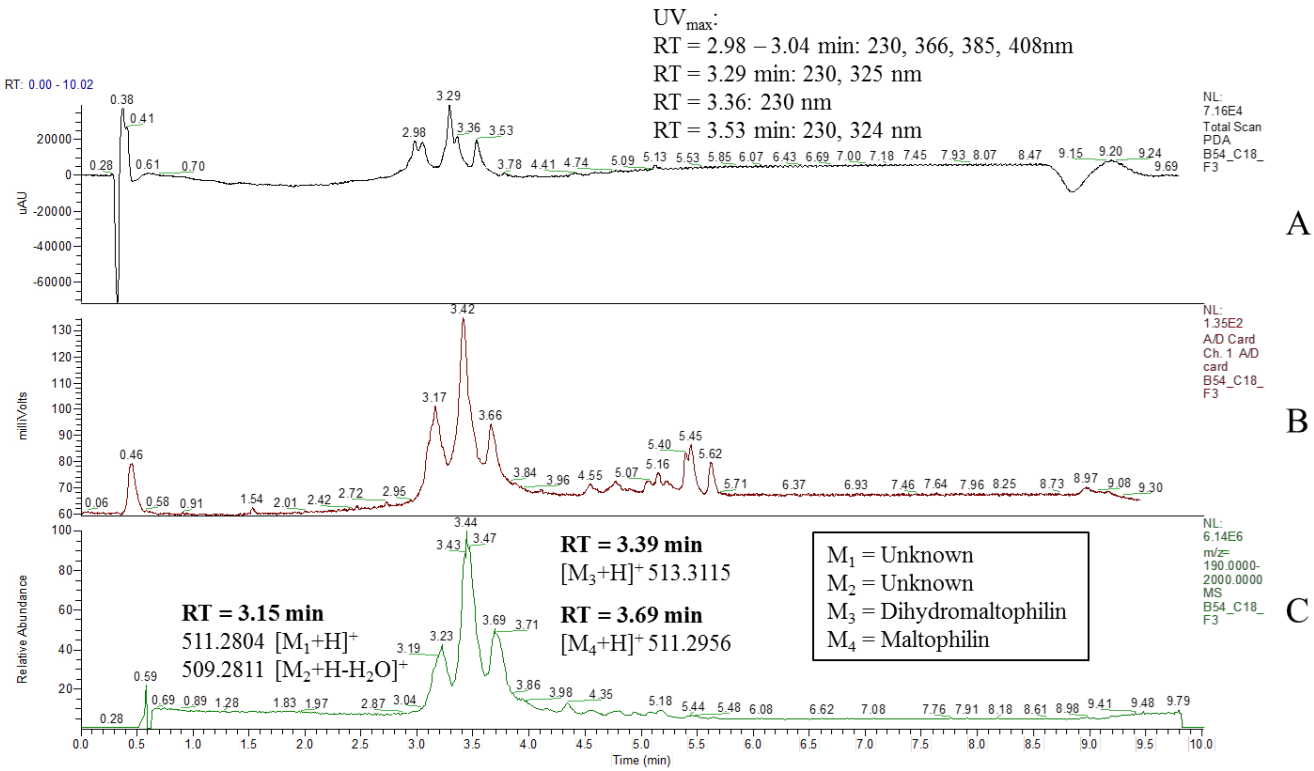


Figure 4.6: UPLC/HRMS profile of RKBH-B54 (C18 Sepak SPE F3) demonstrating A) UV (PDA), including $\lambda_{max(MeOH)}$ values; B) ELSD; C) MS; showing $[M+H]^+$ pseudomolecular ions at highlighted retention times.

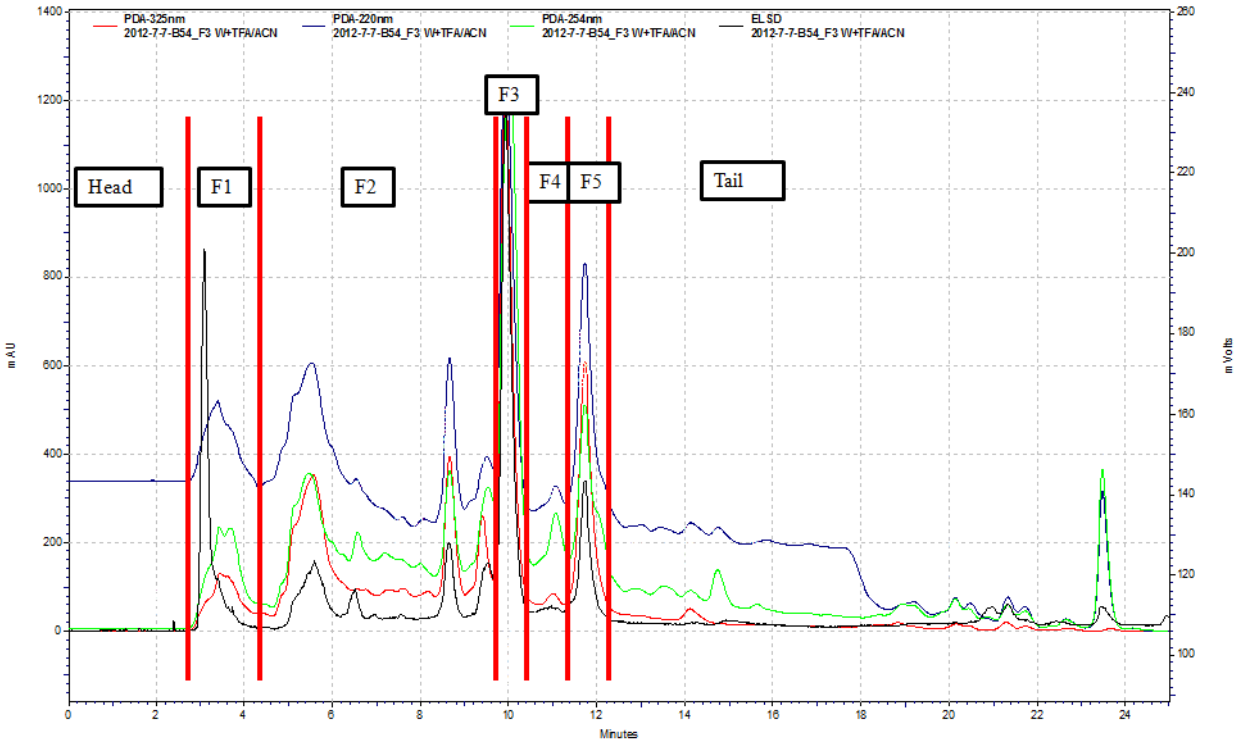


Figure 4.7: HPLC trace of purification Trial 1 of RKBH-B54 – F4. Shown are the collected fractions generated over a 25-minute run. UV (mAU) was monitored at 220 nm (blue), 254 nm (green), and 325 nm (red). The ELSD (mVolts) trace is shown in black.

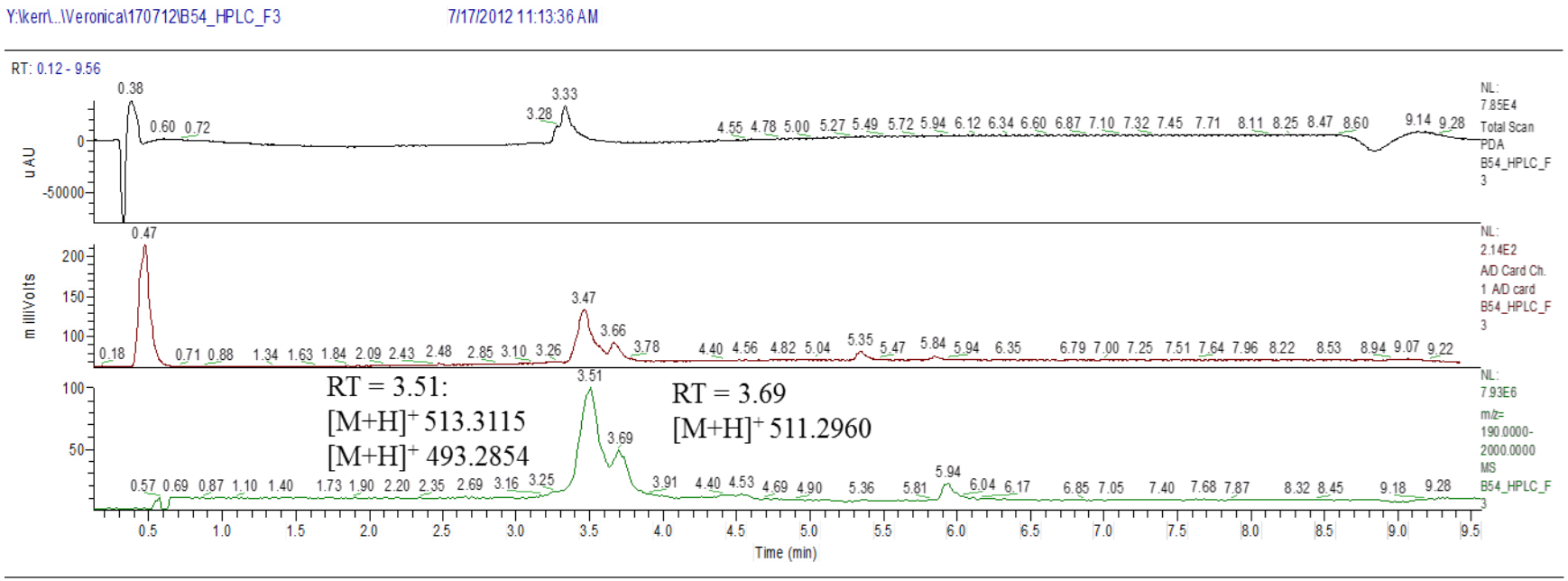


Figure 4.8: UPLC/HRMS profiling of HPLC fraction 3 from RKBH-B54: Trial 1. A) UV; B) ELSD; C) MS showing $[M+H]^+$ pseudomolecular ions. Note the abundance of the polar metabolite(s) at RT 0.47 min in (B).

In total, six fractions were generated: F1: 1.79 mg (yellow powder), F2: 0.66 mg; F3: 2.05 mg; F4: 0.41 mg; F5: 0.28 mg (yellow powder); HPLC Tail: 0.43 mg (Figure 4.9). In this case, UPLC/HRMS profiling revealed that F5 contained compounds with *m/z* 513 $[M+H]^+$ (RT = 3.51 min), 493 $[M+H]^+$ (RT = 3.69 min), and 511 $[M+H]^+$ (RT = 3.69 min; Figure 4.10).

Unfortunately, efforts to purify the antifungal component from RKBH-B54 resulted in apparent compound degradation. Although extensive efforts were taken to ensure sample care (storage of dried extracts at -80°C in glass amber vials), the likely compounds responsible for antifungal activity from RKBH-B54 (*m/z* 513.2955 $[M+H]^+$ and/or 511.2804 $[M+H]^+$) were no longer detected or were detected in low abundance based on UPLC/HRMS following short-term storage and/or RP-HPLC purification (Figure 4.8). However, ELSD and HRMS analysis from HPLC Trial 1 and Trial 2 showed the metabolites at *m/z* 511 and 513 were greatly reduced in quantity following short-term storage or following HPLC purification. Subsequent UPLC/HRMS analyses confirmed this suspicion. For example, metabolites originally abundant based on ELSD were subsequently located in the injection peak at a retention time of 0.46 minutes, which likely was the breakdown product of the proposed tetramic acid (Figure 4.9 – 4.10). In addition, the targeted compounds at *m/z* 511 and 513 (RT = 3.40 min and 3.65 min) displayed a weak detection based on ELSD. However, a molecular formula could not be proposed for this compound as the system is not calibrated to record data until 1 minute following sample injection. Confidence was assumed in suspecting this metabolite was the breakdown product, however; where the possibility that this was salts was eliminated owing to the extensive desalting steps (rinsing with water on two separate occasions followed by C18 SPE) employed in the investigation. The reduction in abundance of these compounds based on ELSD coincided with a

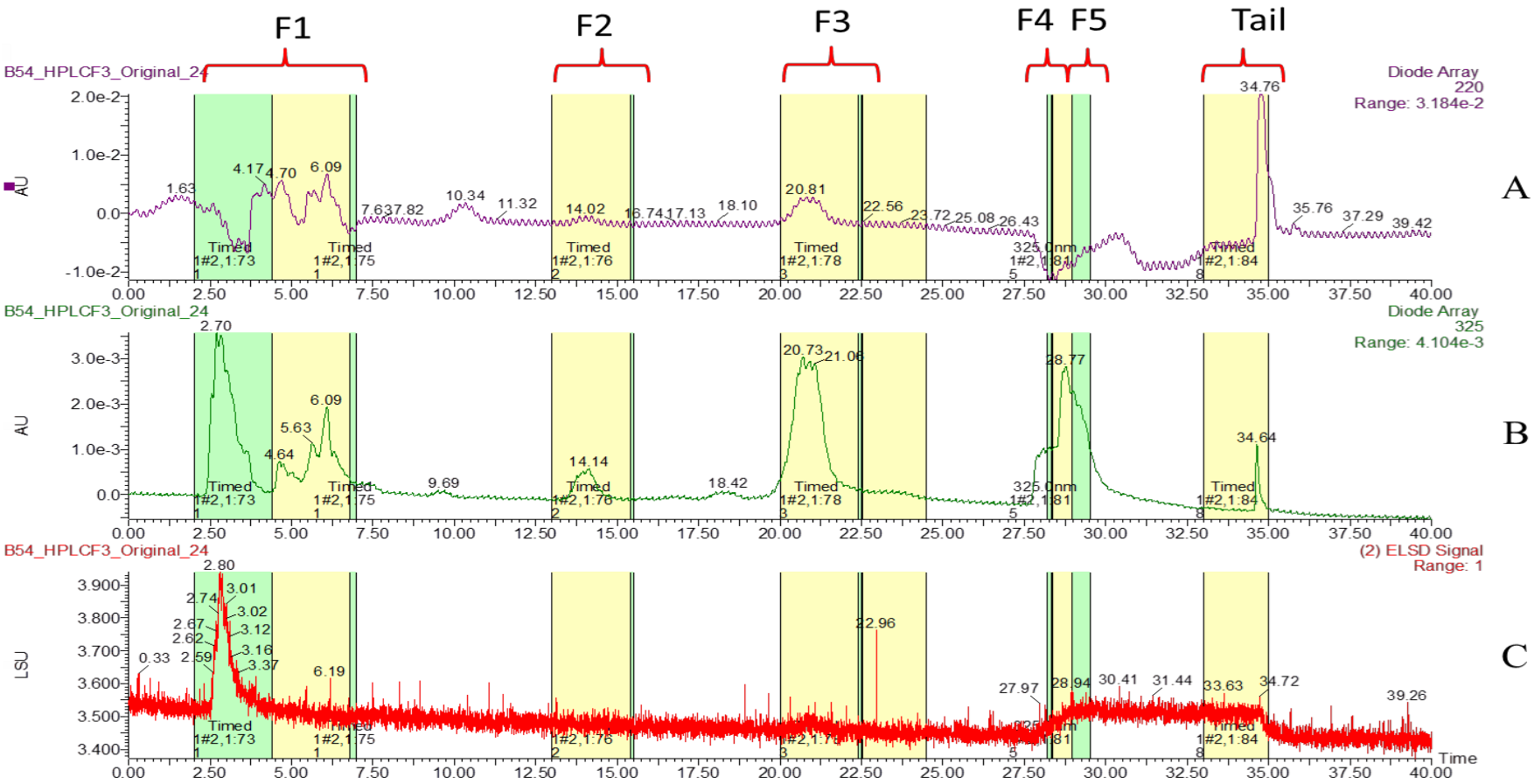


Figure 4.9: HPLC purification of RKBH-B54 100% MeOH extract: Trial 2. Fractions were collected at timed intervals as highlighted above. UV was monitored at (A) 220 nm and (B) 325 nm. ELSD (C) was used to monitor the abundance of each fraction.

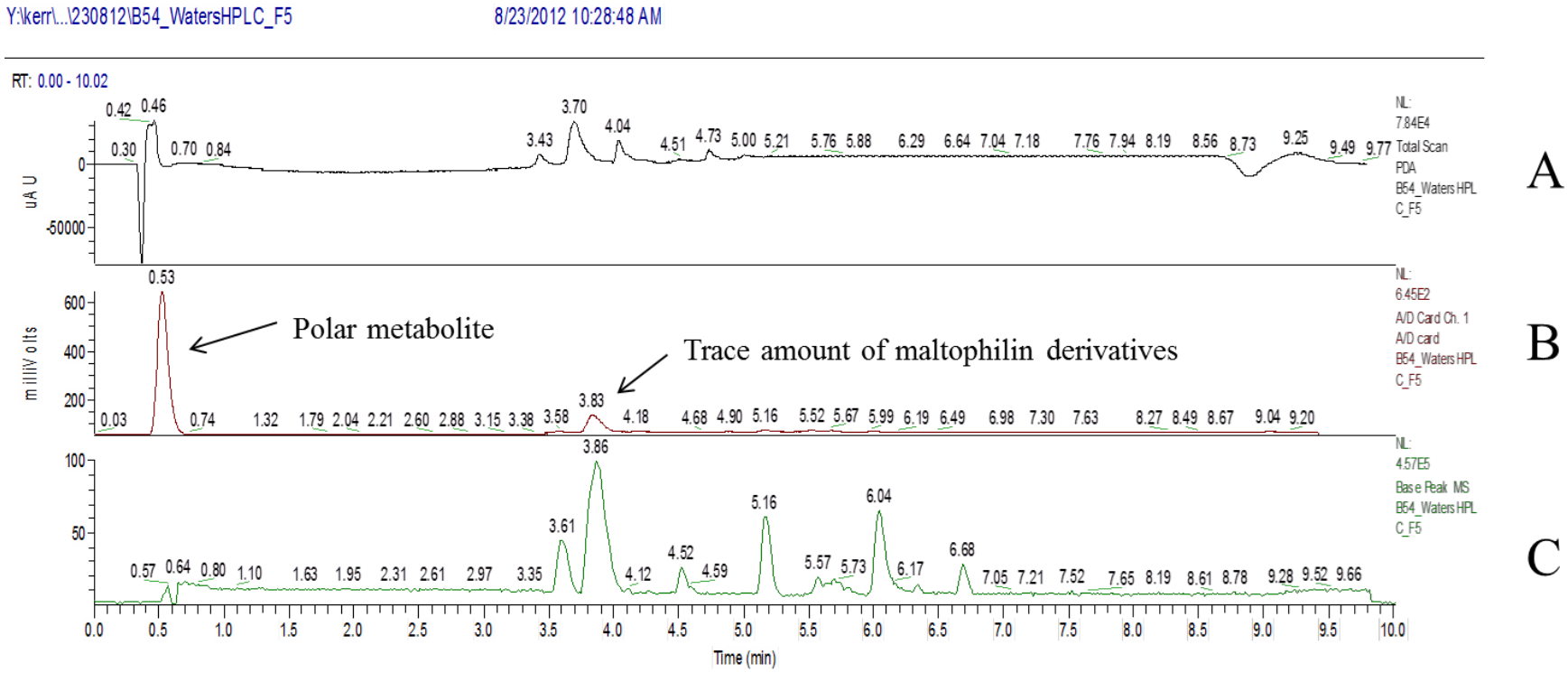


Figure 4.10: UPLC/HRMS profile of HPLC-purified F5 from RKBH-B54. A) UV; B) ELSD; C) MS. Note the reduced abundance of the suspected tetramic acid dihydromaltophilin/maltophilin.

total elimination of biological activity against *C. albicans*. Although the reasoning behind why the antifungal agent degraded so quickly cannot be comprehended at this time, the instability of tetramic acids has been documented previously, both dried and in solution, and appears to involve self-catalyzed aldol condensations (Dittmer *et al*., 2010). In addition, the polyenic chromophore of the related tetramic acid candicidin is sensitive to oxidative degradation (Waksman *et al*., 1965), which may explain the rapid degradation of the compound at least in part. At this stage, there was an insufficient amount of material to continue purification.

Solubility is a recurring theme in the macrolactam class of antifungals

In addition to instability, solubility issues were also encountered when working with this class of metabolite. For example, the compound was soluble only in a mixture of H_2O/MeOH (~1:3) or DMSO and was insoluble in organic solvents (*e.g.* EtOAc, ACN, acetone). Incidentally, solubility was also a factor for dihydromaltophilin (Graupner *et al*., 1997), maltophilin (Jakobi *et al*., 1996), and candicidin (Waksman *et al*., 1965). Each compound was virtually insoluble in all organic solvents, yet the addition of 10 – 15% H_2O to MeOH greatly increased the solubility (Graupner *et al*., 1997; Waksman *et al.,* 1965), a feature which was also observed in the present report. The poor solubility of dihydromaltophilin was attributed to the free base, in addition to the possible presence of conformational isomers in solution (Graupner *et al*., 1997). The insolubility of the compound from RKBH-B54 in organic solvents may also explain some of the difficulty to establish proper HPLC conditions for their purification.

Dihydromaltophilin likely targets fungal sphingolipids by a novel mechanism of action

Structurally, dihydromaltophilin and related compounds belong to an emerging class of polyketide antibiotics containing a macrolactam and a tetramic acid (Graupner *et al*., 1997; Lou *et al*., 2011). Tetramic acids demonstrate a variety of biological activities ranging from antimicrobials, to antivirals, to cytotoxic agents (Lowery *et al*., 2009). Some information regarding their mechanism of action against fungi is known. In particular, dihydromaltophilin, also referred to as heat-stable antifungal factor (HSAF; Li *et al*., 2006), appears to act by a novel mechanism of action which involves inducing cell wall thickening in a diverse array of fungi (Li *et al*., 2009). The thickened cell wall negatively affects fungal hyphal growth, the latter of which is mediated by sphingolipid biosynthesis; therefore, authors suggest that dihydromaltophilin acts via a disruption of the sphingolipid biosynthesis (Li *et al*., 2006). This is significant from a medical standpoint, as fungal sphingolipids appear to be absent or are distinct from mammals and plants and therefore offers an attractive alternative for treating fungal infectious disease (Li *et al*., 2006; Lou *et al*., 2011).

Tetramic acids are produced by a single module hybrid PKS/NRPS

On a molecular level, tetramic acids appear to be produced by an unusual hybrid iterative polyketide synthase-nonribosomal peptide synthase (PKS/NRPS), a feature which is not uncommon in fungi yet was not previously recognized in prokaryotes (Blodgett *et al*., 2010; Lou *et al*., 2011). Through genome mining, Clardy and co-authors determined that this single module hybrid PKS/NRPS was conserved in both Gram positive and Gram negative bacteria, including *Streptomyces* spp. (Blodgett *et al*., 2010) and *Lysobacter enzymogenes* strain C3 (formerly *Stenotrophomonas maltophilia*, Lou *et al*., 2011). Genetic evidence supports the production of

dihydromaltophilin by the PKS/NRPS pathway. For example, the biosynthetic genes involved in dihydromaltophilin contained four open reading frames, including one for a hybrid PKS/NRPS, a sterol desaturase, a ferredoxin reductase, and an arginase. Gene mutants devoid of ferredoxin reductase and arginase maintained the ability to produce dihydromaltophilin with no real impact on yield. Conversely, sterol desaturase-disrupted mutants did not contain dihydromaltophilin, yet produced two other metabolites which were active in antifungal assays (Yu *et al*., 2007). However, dihydromaltophilin could no longer be produced in PKS/NRPS-disrupted mutants (Yu *et al*., 2007).

Bioactive tetramic acids have been identified from a variety of organisms, including *Streptomyces* spp. isolated from various terrestrial and marine sources including Attine ants (Blodgett *et al*., 2010; Haeder *et al*., 2009; Seipke *et al*., 2011), soil (Graupner *et al*., 1997), the plant-derived bacteria *Stenotrophomonas maltophilia* (Jakobi *et al*., 1997), and the marine sponge *Discodermia dissoluta* (Gunasekera *et al*., 1990). The wide distribution of tetramic-acid containing antifungal compounds in a diverse selection of microorganisms suggests that these molecules are an important group of compounds involved in microbial interactions (Li *et al*., 2009). Furthermore, the report of a marine-isolated *Streptomyces* sp. described herein to likely produce bioactive tetramic acid(s) develops intriguing questions: was this isolate washed from the terrestrial environment into the sea, ingested by the coral accidentally, and maintained owing to its antifungal benefits? This hypothesis, where marine bacteria may be of terrestrial origin, has been addressed previously (Jensen and Fenical, 1994) and this report has provided evidence that a marine-isolated *Streptomyces* sp. is likely capable of producing similar antifungal compounds to those reported from terrestrial-isolated *Streptomyces* sp. This thesis is the first report to document that a bacterium isolated from an octocoral may be producing maltophilin derivatives.

CONCLUSIONS AND SIGNIFICANCE

This thesis has demonstrated that Gammaproteobacteria and Actinobacteria isolated from the Bahamian octocoral *P. elisabethae* are capable of selectively and reproducibly inhibiting pathogenic Gram positive bacteria and fungi respectively. By incorporating salt and HP-20 resin, in fermentation media, the results have shown that the likelihood of detecting antimicrobial activity in fermentation extracts increased. Specifically, BFM3 and mMB + HP-20 were recommended as media to utilize in subsequent fermentations owing to the higher number of isolates with bioactivity when fermented in these media. Specifically, several genetically and chemically related *Pseudoalteromonas* spp. were cultured from *P. elisabethae*, where fermentation extracts were active against the Gram positive organisms MRSA and VRE. Fermentation extracts from *Streptomyces* sp. RKBH-B54 also demonstrated significant antifungal activity against *C. albicans*. After comparing UV spectra, HRMS data, and following bioassay guided fractionation, chemical profiling revealed that the most likely antifungal compound(s) produced by *Streptomyces* sp. RKBH-B54 were a series of related tetramic acids, notably dihydromaltophilin and maltophilin. However, owing to its rapid degradation, efforts to purify the antifungal component were unsuccessful.

This investigation has led to the formulation of several additional questions, notably resolving around the perceived compound degradation – what measures can be taken to improve the stability of the compound? Future investigations can expand on the biological activities of coral-associated bacteria by including assays such as those which test for anti-algal activity, probiotic activity, or anticancer activity. In addition, the possible ecological role of *Streptomyces* sp. RKBH-B54 in the host could be addressed by testing extracts for activity against *Aspergillus sydowii*, the fungal pathogen which appears to be responsible for causing coral mortality via

aspergillosis (Alker, 2001; Rypien, 2008). Previous investigations have shown that dihydromaltophilin is capable of inhibiting *Aspergillus nidulans*, and since dihydromaltophilin was suspected to be an isolated product from *Streptomyces* sp. RKBH-B54, it is highly likely that the compound isolated in this investigation demonstrates additional bioactivity against marine fungal pathogens. Although this report has provided evidence that bacteria isolated from *P. elisabethae* are capable of producing antimicrobial agents, whether or not the natural product stimulation is produced in the coral's natural habitat is yet to be understood.

Finally, feeding experiments are underway in the Kerr lab to determine if RKBH-B54 titers can be increased by the addition of the amino acid ornithine, which is naturally incorporated into the lactam in dihydromaltophilin (Lou *et al*., 2011). Ornithine appears to be a significant feature involved in the biosynthesis of dihydromaltophilin and is also critical in antifungal activity (Lou *et al*., 2011; Li *et al*., 2012). Specifically, the hydroxyl moiety in the residue at the C3 position appears to be vital to bioactivity, as knock-out studies of the sterol desaturase gene (involved in 3-hydroxylation) demonstrated that HSAF lost antifungal activity in the mutant compound (Li *et al*., 2012). However, although the significance of the ornithine residue in antifungal activity has been established, it is not known whether the yield of the antifungal compound can be improved by the adding ornithine to the fermentation media. Hence, investigations of increasing the supply of the antifungal agent can dramatically affect the possibility of marketing the compound through clinical trials.

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CHAPTER V

CONCLUSIONS AND PERSPECTIVES

The main objective of this study was to investigate the bacterial diversity and the biomedical potential of natural products derived from bacterial fermentation extracts from the alcyonacean octocoral *Pseudopterogorgia elisabethae*. To achieve this goal, three studies were performed: 1) the bacterial community was analyzed using culture-independent next generation pyrosequencing, 2) the cultivable bacterial diversity from *P. elisabethae* was assessed using a diverse selection of isolation media to maximize the number of phylogenetically unique isolates, and 3) fermentation extracts from the cultivable bacterial community were analyzed for natural products via antimicrobial screening, followed by bioassay-guided purification of an antifungal compound(s).

Despite the plethora of information regarding scleractinian-associated bacterial diversity, very little is known regarding bacterial diversity in octocorals. Specifically, no investigation has studied the bacterial community of the commercially important octocoral *P. elisabethae*. Consequently, the **first research objective** sought to answer the following questions: is the bacterial diversity of *P. elisabethae* conserved within individuals on the same reef? How comparable is the bacterial community between individuals across reefs and throughout the Bahamas, and how do these communities compare to the surrounding seawater? Based on general findings from scleractinian-associated bacteria, this thesis hypothesized that the bacterial community of *P. elisabethae* would be conserved both within and between reefs in the Bahamas, yet would be distinct from the surrounding seawater as other studies have suggested (Rohwer *et al*., 2001; Sweet *et al*., 2011). To answer these questions, coral-associated bacterial communities

were analyzed by bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) from triplicate corals each collected from four regions in the Bahamas.

The results were unexpected: the 16S rRNA gene sequence community in *P. elisabethae* individuals was variable both at the class ($OTU_{0.10}$) and at the species level ($OTU_{0.03}$) as demonstrated by community composition and principal component analysis respectively. Contrary to the hypothesis, these results clearly suggested that bacterial communities were distinct both within and across reefs in the Bahamas, and therefore suggest that the coral may be obtaining bacteria which offer distinct physiological advantages in the local environment. In general, however, Gammaproteobacteria, Cyanobacteria, and Alphaproteobacteria were consistently dominant in both coral and water-associated bacterial libraries, while an unclassified bacterial phylum phylotype was particularly abundant in gDNA-derived pyrosequencing libraries. Interestingly, Alphaproteobacteria appeared to be the most phylogenetic diverse at the species level, while Gammaproteobacteria, Cyanobacteria, and Flavobacteria were dominated by closely related phylotypes. Dominant $OTU_{0.03}$s in bTEFAP did not necessarily reflect the most dominant DGGE retrieved band sequences, however, and did not reflect the numerical abundance of Alphaproteobacteria or Gammaproteobacteria as seen in bTEFAP sequence libraries, although the most dominant retrieved bands were most closely related to *Prochlorococcus marinus* (NR028762.1)/*Synechococcus* sp. However, one DGGE band sequence corresponded to the bTEFAP sequence RKPE23, which due to its low sequence identity to its nearest cultured GenBank representative (80% sequence identity to *Mycoplasma alkalescens*) may represent a candidate bacterial phylum. Interestingly, RKPE23 was most closely related to an uncultured phylotype from the closely related octocoral *Gorgonia ventalina* (Gven_K23, 98% sequence identity). These appeared to be novel, octocoral-specific bacterial

phylotypes which have as-of-yet no cultured representative. In addition, the phylotype RKPE1 may represent a candidate bacterial class, which shared only 91% sequence homology to the reference strain *Endozoicomonas elysicola*. Similarly, this phylotype was most similar (98% sequence identity) to a *G. ventalina* clone RKPE1. These phylotypes (RKPE1 and RKPE23) were particularly abundant in gDNA-derived bacterial pyrosequencing libraries from *P. elisabethae*. These phylotypes were not detected in the surrounding seawater. Based on the apparent novelty of these phylotypes, which have only been reported from two other octocorals *G. ventalina* and *Eunicella cavolini* (Sunagawa *et al.*, 2010; unpublished), the first research objective demonstrated that *P. elisabethae* provides a uniquely specialized habitat for potentially novel groups of marine bacteria. Furthermore, based on the highly variable biological diversity of the associated prokaryotic community, it was proposed that *P. elisabethae* associated bacteria offers a new and diverse resource for natural product drug discovery.

Based on the discovery that *P. elisabethae* harbours a diverse prokaryotic community, the **second research goal** aimed to cultivate *P. elisabethae*-associated bacteria. The goal in cultivating coral associated bacteria was to generate an isolate library suitable for subsequent drug discovery. This thesis hypothesized that a diverse selection of media, in combination with the particle filtration technique, would aid in increasing the number of phylogenetically unique cultivable bacteria. Furthermore, the objective was to use MALDI-TOF MS as a means to dereplicate cultivable isolates, a methodology which was previously conducted on sponge-associated bacteria but had not yet been published as a tool to dereplicate coral associated bacteria. It was hypothesized that MALDI-TOF MS would be a reliable and rapid means with which to dereplicate coral-associated bacterial isolates from *P. elisabethae*. Finally, the cultured

bacterial community of *P. elisabethae* was compared to the culture-independent bacterial community to determine the overlap of bacteria using molecular and traditional techniques.

The aerobic cultivation efforts on fresh and frozen *P. elisabethae* homogenates yielded a total of approximately 826 bacterial isolates from three collection sites and two collection times. Owing to its rapidity, ease of use, and ability to reliably differentiate closely related isolates, it was demonstrated that whole-cell bacterial in-tact MALDI-TOF MS could be successfully used as a tool to dereplicate octocoral-associated bacteria. Numerically, Gammaproteobacteria represented the most abundant isolates at the class level corresponding to 55% of the total isolates retrieved, followed by Alphaproteobacteria (17%), Bacilli (11%), Actinobacteria (10%), and Flavobacteria (2%). In addition, the cultivation efforts yielded seven potentially novel bacterial genera based on BLAST sequence comparisons to characterized 16S rRNA gene sequences, thus confirming the hypothesis that *P. elisabethae* harbors unique bacterial species. According to the phylogenetic analysis, however, cultures obtained from fresh coral homogenate were biased towards Gram negative bacteria, while isolation efforts from frozen coral homogenate were more selective for isolating low GC and Gram positive bacteria. Based on these results, it was proposed that freezing coral homogenate prior to plating adds a unique benefit in increasing the number of phylogenetically distinct bacteria, allowing for the selective isolation of low GC and Gram positive bacteria. Conserved between year, site, and homogenate type (fresh vs. frozen) were isolates most closely related to *Bacillus* sp., *Micrococcus* sp., and *Pseudomonas* sp. (Figure 3.5). Their presence between collection time and across sites in the Bahamas indicates that these phylotypes may be important symbiotic members in the *P. elisabethae* holobiont.

Sequences generated from dereplicated isolates were clustered at $OTU_{0.03}$ with pyrosequencing data, where it was hypothesized that all isolate sequences would have a comparable $OTU_{0.03}$ generated by pyrosequencing. Although difficulties in DNA extractions prevented pyrosequencing of the majority of 2009-collected corals, a comparison of *P. elisabethae* cultures to pyrosequence data provides a general overview of the overlap of the bacterial communities described by these two techniques. Cross-correlation of bTEFAP sequences to bacterial isolate sequences revealed a 0.44% overlap of cultures to bTEFAP sequences at $OTU_{0.03}$. Hence, the results clearly demonstrated bacterial diversity was underrepresented by cultivation. Surprisingly, the cultivable bacterial community of *P. elisabethae* demonstrated that Alphaproteobacteria were almost exclusively represented by *Ruegeria* sp., Gammaproteobacteria were represented by a more diverse group of cultured representatives (*Alteromonas* sp., *Pseudoalteromonas* sp., and *Vibrio* sp.), while no cultured representatives of Cyanobacteria were detected. This result contrasted with the bTEFAP analysis, which demonstrated Alphaproteobacteria represented the highest phylotype diversity while Gammaproteobacteria and Cyanobacteria were comprised of several closely-related phylotypes at the species level. Furthermore, the most abundant cultures from *P. elisabethae* did not have a comparable bTEFAP representative sequence. For example, although *Vibrio* spp. dominated the cultivable bacterial community, many did not have a comparable $OTU_{0.03}$ in the pyrosequencing data generated from 14 corals. No octocoral-specific bacteria (*e.g.* RKPE1 and RKPE23-related phylotypes) were cultured using the methodologies described in this thesis. Although it is unusual that the most abundant bacteria from bTEFAP did not overlap with the most numerous bacteria from cultivation, the lack of congruence between culture-dependent and molecular techniques is a trend which has been repeatedly documented in the literature

(Galkiewicz *et al*., 2011; Gray *et al*., 2011; Meron *et al*., 2011), affirming that using both techniques is vital to uncovering the true bacterial diversity. From the second research objective, it was concluded that not only was MALDI-TOF MS able to suitably dereplicate coral-associated bacteria, but the discovery of potentially novel cultivable bacterial species suggested that *P. elisabethae*-associated bacteria are a unique resource for natural product investigations. Isolates were also screened against pseudopterosin G (PsG) with the aim of determining the antimicrobial activity against ecologically relevant bacteria residing within the coral. This is due to the fact that pseudopterosins from Colombian and Bahamian collected *P. elisabethae* have selective activity against Gram positive bacteria (Ata *et al*., 2004; Correa *et al*., 2011). This investigation revealed that while Gram negative bacteria were largely unaffected by PsG, Gram positive bacteria were highly susceptible, where the growth of 88% of Gram positive bacteria were inhibited. In addition, isolates were tested for salinity requirements to add to the scientific understanding of bacteria isolated from marine invertebrates which are dependent on seawater for growth. These results suggested Gram negative bacteria were far more likely to require seawater to remain viable than Gram positive bacteria.

Although the octocoral *P. elisabethae* is a well-characterized source of antibacterial diterpenes (Ata *et al*., 2004; Correa *et al*., 2009), its associated microbial community has not been assessed for natural product production. Results from objective two led to evaluating the ability of *P. elisabethae*-associated bacteria to produce secondary metabolites. Hence, the **third research objective** was to discover new antimicrobial agents from coral-associated bacterial extracts. It was theorized that isolates from *P. elisabethae* will be unique to the host, and therefore the likelihood that finding similar microorganisms from another host will be greatly reduced. The ability of coral associated bacteria to produce natural products was assessed by

testing bacterial extracts for antimicrobial activity against a panel of clinically relevant pathogens.

As expected, the majority of isolates affected by the addition of PsG to the media were Gram positive, with 44.7% of Gram positive isolates inhibited while 18.4% were sensitive at 50 mg/L PsG, while Gram negative bacteria were largely unaffected at all PsG concentrations tested. In contrast, the majority of isolates (57.1%) from *P. elisabethae* were considered "marine", based on their dependency on MA (salt-containing media) for growth. Interestingly, the results suggested that Gram negative bacteria were more likely to require sodium chloride for successful growth and viability, where 86.8% of Gram negative isolates required salt for growth in comparison to only 28.9% of Gram positive bacteria. These studies have increased our understanding of the ability of natural products to affect particular associated bacteria and have further enhanced our knowledge regarding salinity dependencies of bacteria isolated from marine sources.

In the investigation on coral-associated bacterial extracts demonstrating antimicrobial activity, the results showed that *Pseudoalteromonas* sp. RKVR-02, RKVR-07, RKVR-20, and RKVR-34 and *Streptomyces* sp. RKBH-B54 were capable of selectively and reproducibly inhibiting pathogenic Gram positive bacteria and fungi respectively. Acetonitrile extracts from the chemically and genetically related *Pseudoalteromonas* sp. (RKVR-02, RKVR-07, and RKVR-34) demonstrated reproducible inhibition against the Gram positive bacteria MRSA and VRE when grown in M11. *Pseudoalteromonas* sp. RKVR20 (maroon pigmented) demonstrated moderate activity against MRSA when extracted from seed media (MB), yet extracts were inactive when grown in fermentation media.

Owing to its potent and reproducible antifungal activity against *Candida albicans*, RKBH-B54 was prioritized for large scale fermentation and bioassay guided fractionation. After comparing UV spectra and HRMS data, chemical profiling revealed that the most likely antifungal compound(s) produced by *Streptomyces* sp. RKBH-B54 belonged to related tetramic acids. For example, the pseudomolecular ion at 513.29547 $[M+H]^+$ was a match to dihydromaltophilin, an antifungal compound previously reported from a soil-inhabiting *Streptomyces* sp. with the molecular formula $C_{29}H_{40}N_2O_6$ (Graupner *et al*., 1997). Secondly, the pseudomolecular ion at *m/z* 511.29651 $[M+H]^+$ was a match to maltophilin (Jakobi *et al*., 1996), a compound reported from a Gram negative bacterium. However, reverse-phase HPLC of these related metabolites was difficult owing to their virtually identical retention times. Furthermore, despite extensive efforts in sample care, the compound(s) likely responsible for the observed antifungal activity in RKBH-B54 appeared to have degraded over time, and eventually the compounds most likely causing bioactivity were no longer detected or were detected in low abundance based on ELSD following short-term storage and/or RP-HPLC purification. All antifungal activity was lost following the disappearance/reduction of these compounds.

Owing to the highly potent anti-fungal activity caused by extracts of RKBH-B54, this bacterium was targeted for additional experiments. Feeding experiments are currently underway in the Kerr lab to determine if titres of the antifungal compound can be increased by the addition of the amino acid ornithine to the fermentation media. Ornithine is an essential precursor to dihydromaltophilin biosynthesis, with the hydroxyl moiety being critical in ensuring antifungal activity (Li *et al*., 2012). Although it is clear that ornithine is incorporated into the macrolactam unit, no study has performed detailed analysis to determine whether yields can be improved by supplementing the bacterium in media containing ornithine. It is also rationalized that feeding

arginine to the fermentation media may increase the yield of the antifungal compound, as ornithine is generated from the action of the enzyme arginase from arginine (Lou *et al*., 2011). Similarly, it may be possible to generate novel analogs of dihydromaltophilin by feeding ornithine analogs such as L-lysine, which contains an additional CH_2 group in the carbon skeleton. Preliminary evidence suggests that supplementing fermentation media with 0.5 mg/mL ornithine can significantly improve the yields of the antifungal compound by up to four times.

In summary, the present study has investigated the bacterial community and biomedical activities of bacterial extracts from *P. elisabethae* and has demonstrated that this coral contains an unexpectedly diverse bacterial community. Surprisingly, bacterial communities of *P. elisabethae* individuals were variable within and between reefs in the Bahamas, which were generally not comparable between members at both the species level ($OTU_{0.03}$) and the class level ($OTU_{0.10}$). Fermentation extracts from select members demonstrated antimicrobial activity against clinically relevant pathogens. This thesis has contributed to the knowledge of octocoral-associated bacterial communities, which has received very little attention in the literature. In addition, detailed bacterial species-level classification for dominant pyrosequences have been provided which revealed that many closely-related, likely similar phylotypes were retrieved. Consequently, future investigators may consider using a less conservative taxonomic clustering at $OTU_{0.04}$ as proposed by Kim *et al*. (2011). In addition, this study characterized the antimicrobial potential of fermentation extracts from *Pseudoalteromonas* spp. and *Streptomyces* sp. isolated from *P. elisabethae*, which were bioactive against Gram positive bacteria and yeast respectively. Future studies may benefit from the information acquired from this thesis in a variety of ways. For example, the data generated from this investigation can be used to compare to other investigations of octocoral and stony coral-associated bacteria. This knowledge can be

used to guide culturing programs, as knowing which bacteria are present can assist targeting efforts for specific groups of bacteria. In addition, determining if the *P. elisabethae* isolates *Pseudoalteromonas* sp. and *Streptomyces* sp. are capable of producing their respective antimicrobial compounds *in vivo* would also suggest the ecological relevance of these bacteria in protecting the coral host. On-going experiments are currently being conducted in the Kerr laboratory to determine the location of bacteria in *P. elisabethae*.

This study raises some intriguing biological questions. Why is the intra-individual variability so high in bacteria associated with *P. elisabethae,* and is this a trend which extends to other octocorals? How can we alter the global paradigm which states that less than one percent of bacteria can be cultured, and what information is vital to increase our ability to cultivate microbes? Do the cultures isolated from *P. elisabethae* produce natural products which have additional biological activities which may have been overlooked in the present investigation? Answers to such questions can contribute to our understanding of octocoral-associated bacterial communities and can provide a resource for discovering a sustainable supply of novel natural products.

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APPENDIX A

Table A1: Accession numbers and nearest GenBank representatives for RKPE sequences from bTEFAP libraries.

RKPE sequence	Genbank Acc No	Description	Closest GenBank Representative	Percent similarity
RKPE1	JN411801.1	Uncultured gamma proteobacterium clone RKPE1	Uncultured bacterium clone Gven_G10 (GU118518.1)	99
RKPE2	JN411784.1	Uncultured bacteroidetes bacterium clone RKPE2	*Aquimarina* sp. PSC32 (HM998909.1)	95
RKPE3	JN411758.1	Uncultured cyanobacterium clone RKPE3	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE4	JN411799.1	Uncultured proteobacterium clone RKPE4	Uncultured bacterium clone Gven_G10 (GU118518.1)	99
RKPE5	JN411756.1	Uncultured cyanobacterium clone RKPE5	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE6	JN411722.1	Uncultured proteobacterium clone RKPE6	Uncultured bacterium isolate DGGE gel band 2 (JN863712.1)	99
RKPE7	JN411768.1	Uncultured cyanobacterium clone RKPE7	*Synechococcus* sp. KORDI-28 (FJ497720.1)	98
RKPE8	JN411803.1	Uncultured gamma proteobacterium clone RKPE8	Uncultured bacterium clone Gven_PO4 (GU118455.1)	99
RKPE9	JN411770.1	Uncultured cyanobacterium clone RKPE9	Uncultured *Prochlorococcus* sp. clone 97 (JN547456.1)	99
RKPE10	JN411781.1	Uncultured bacteroidetes bacterium RKPE10	Uncultured Flavobacteriaceae bacterium clone GG101008	91
RKPE11	JN796467.1	Uncultured alpha proteobacterium clone RKPE11	*Sphingobium rhizovicinum* strain CC-FH12-1 (NR_044226.1)	98
RKPE12	JN796464.1	Uncultured alpha proteobacterium clone RKPE12	*Ruegeria* sp. G-M8 (JQ807219.1)	98
RKPE13	JX266009.1	Uncultured bacterium clone RKPE13	Uncultured bleached octocoral Rhodospirillaceae bacterium clone BME47	99
RKPE14	JN411798.1	Uncultured gamma proteobacterium clone RKPE14	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE15	JN411757.1	Uncultured cyanobacterium clone RKPE15	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE16	JN411771.1	Uncultured cyanobacterium clone RKPE16	Uncultured *Prochlorococcus* sp. clone 68 (JN547449.1)	99
RKPE17	JN411775.1	Uncultured cyanobacterium clone RKPE17	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE18	JN411766.1	Uncultured cyanobacterium clone RKPE18	Uncultured bacterium clone Gven_N16 (GU118510.1)	99
RKPE19	JN796447.1	Uncultured alphaproteobacteria bacterium RKPE19	Uncultured *Brevundimonas* sp. (FR657544.1)	99
RKPE20	JN411794.1	Uncultured gamma proteobacterium clone RKPE20	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE21	JN411795.1	Uncultured proteobacterium clone RKPE21	Uncultured bacterium clone Gven_G10 (GU118518.1)	97
RKPE22	JN796452.1	Uncultured alpha proteobacterium clone RKPE22	Uncultured bacterium clone BT60DS1BD3 (AF365701.1)	99
RKPE23	JN411746.1	Uncultured bacterium clone RKPE23	Uncultured octocoral associated bacterium clone Gven_K23 (GU118339.1); Uncultured bacterium isolate DGGE gel band 1 (JN863711.1)	100

RKPE24	JN411767.1	Uncultured cyanobacterium clone RKPE24	Uncultured bacterium clone Gven_N16 (GU118510.1)	98
RKPE25	JN411786.1	Uncultured proteobacterium clone RKPE25	Uncultured *P. elisabethae* bacterium clone PEA10 (JQ609313.1)	98
RKPE26	JN796444.1	Uncultured alpha proteobacterium clone RKPE26	Uncultured *P. elisabethae* bacterium clone PEA09 (JQ609307.1)	98
RKPE27	JN411788.1	Uncultured proteobacterium clone RKPE27	Uncultured coral-associated bacterium clone Apal_F24 (GU118110.1)	93
RKPE28	JN411747.1	Uncultured bacterium clone RKPE28	Uncultured bacterium clone Cyano2A11 (EU917873.1)	94
RKPE29	JN411763.1	Uncultured bacterium clone RKPE29	Uncultured bacterium clone PoritesC32clB02 (EU636615.1)	97
RKPE30	JN411796.1	Uncultured gamma proteobacterium clone RKPE30	Uncultured bacterium clone Gven_G10 (GU118518.1)	99
RKPE31	JN411759.1	Uncultured cyanobacterium clone RKPE31	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE32	JN411802.1	Uncultured gamma proteobacterium clone RKPE32	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE33	JN411772.1	Uncultured cyanobacterium clone RKPE33	*Prochlorococcus marinus* subsp. pastoris str. PCC 9511 (NR_028762.1)	97
RKPE34	JN796462.1	Uncultured alpha proteobacterium clone RKPE34	*Ruegeria* sp. MA1-6 (HQ852038.1)	99
RKPE35	JN796455.1	Uncultured alpha proteobacterium clone RKPE35	Uncultured alpha proteobacterium (FM211779.1)	98
RKPE36	JN411748.1	Uncultured bacterium clone RKPE36	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE37	JN411724.1	Uncultured proteobacterium clone RKPE37	*Pelagibius litoralis* strain CL-UU02 (NR_043785.1)	97
RKPE38	JN411740.1	Uncultured actinomycetales bacterium clone RKPE38	*Mycobacterium tuberculosis* (NR_044826.1); Uncultured bacterium isolate DGGE gel band 4 (JN863714.1)	99
RKPE39	JN411726.1	Uncultured proteobacterium clone RKPE39	Uncultured bacterium isolate DGGE gel band 2 (JN863712.1)	99
RKPE40	JN411783.1	Uncultured bacteroidetes bacterium clone RKPE40	*Aquimarina* sp. PSC32 (HM998909.1)	94
RKPE41	JN411774.1	Uncultured cyanobacterium clone RKPE41	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE42	JN411760.1	Uncultured cyanobacterium clone RKPE42	*Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE43	JN411761.1	Uncultured cyanobacterium clone RKPE43	Uncultured bacterium isolate DGGE gel band 3, 5, 7, PE3C (JN863718.1); *Synechococcus* sp. KORDI-54 (FJ497735.1)	99
RKPE44	JN411728.1	Uncultured alpha proteobacterium clone RKPE44	Uncultured juvenile acroporid coral associated bacterium clone MC3F09	99
RKPE45	JN796460.1	Uncultured alpha proteobacterium clone RKPE45	*Paracoccus zeaxanthinifaciens* strain R-1506 (AF461159.1)	97
RKPE46	JN796446.1	Uncultured alpha proteobacterium clone RKPE46	*Brevundimonas terrae* strain C-G-NA6 (HM755493.1)	99
RKPE47	JN796459.1	Uncultured alpha proteobacterium clone RKPE47	Mucus bacterium from an acroporid coral (AY654761.1)	99
RKPE48	JN411792.1	Uncultured gamma proteobacterium clone RKPE48	*Pseudomonas putida* (AB680123.1)	99

RKPE49	JN411782.1	Uncultured bacteroidetes bacterium clone RKPE49	*Aquimarina* sp. PSC32 (HM998909.1)	92
RKPE50	JN411773.1	Uncultured cyanobacterium clone RKPE50	Uncultured reef water associated bacterium clone Reef_I17	99
RKPE51	JN411729.1	Uncultured proteobacterium clone RKPE51	Uncultured SAR11 cluster alpha proteobacterium clone PRTAB7855	100
RKPE52	JN411764.1	Uncultured cyanobacterium clone RKPE52	Uncultured acroporid associated bacterium clone Mfra_D07	95
RKPE53	JN796448.1	Uncultured alpha proteobacterium clone RKPE53	Uncultured *P. elisabethae* bacterium clone PEA08	99
RKPE54	JN796449.1	Uncultured alpha proteobacterium clone RKPE54	*Brevundimonas vesicularis* strain: NBRC 12165 (AB680247.1)	99
RKPE55	JX266012.1	Uncultured bacterium clone RKPE55	Uncultured marine water-associated bacterium clone HglFeb6H9m	99
RKPE56	JN796468.1	Uncultured alpha proteobacterium clone RKPE56	*Rickettsia rhipicephali* strain 3-7-6 (NR_025921.1)	99
RKPE57	JN411727.1	Uncultured alpha proteobacterium clone RKPE57	Uncultured bacterium clone Dstr_H14 (GU118150.1)	99
RKPE58	JN411769.1	Uncultured cyanobacterium clone RKPE58	*Synechococcus* sp. KORDI-28 (FJ497720.1)	98
RKPE61	JN796445.1	Uncultured alpha proteobacterium clone RKPE61	*Mycoplana bullata* strain IAM 13153 (NR_025831.1)	98
RKPE63	JN411797.1	Uncultured gamma proteobacterium clone RKPE63	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE64	JN411785.1	Uncultured bacteroidetes bacterium clone RKPE64	*Aquimarina intermedia* strain LMG 23204 (NR_042444.1)	94
RKPE67	JN796465.1	Uncultured alpha proteobacterium clone RKPE67	*Ruegeria* sp. UloCh-03	97
RKPE68	JN411725.1	Uncultured proteobacterium clone RKPE68	Uncultured alpha proteobacterium clone SIMO-1536 (AY710976.1)	98
RKPE72	JN796450.1	Uncultured alpha proteobacterium clone RKPE72	*Brevundimonas vesicularis* (AB680247.1)	98
RKPE73	JN411733.1	Uncultured alpha proteobacterium clone RKPE73	Uncultured coral associated bacterium clone Mfra_E05 (GU118700.1)	99
RKPE78	JN411734.1	Uncultured alpha proteobacterium clone RKPE78	Uncultured bacterium clone 47-S-68 (JN018674.1)	99
RKPE99	JN411730.1	Uncultured bacterium clone RKPE99	Uncultured South Atlantic bacterium clone ST13_70m (HQ015557.1)	98
RKPE81	JN411735.1	Uncultured bacterium clone RKPE81	Uncultured coral-associated bacterium clone Past_N22 (GU118960.1)	95
RKPE82	JN411723.1	Uncultured proteobacterium clone RKPE82	Uncultured Bahamian thrombolite proteobacterium clone Pink_2F06 (GQ483965.1)	97
RKPE84	JN796461.1	Uncultured alpha proteobacterium clone RKPE84	Uncultured coral reef sand bacterium clone Si06 94	99
RKPE87	JN411741.1	Uncultured actinomycetales bacterium clone RKPE87	*Mycobacterium tuberculosis* (NR_044826.1); Uncultured bacterium isolate DGGE gel band 4 (JN863714.1)	99
RKPE88	JN411732.1	Uncultured bacterium clone RKPE88	Uncultured bacterium clone S23_1425	99
RKPE90	JN796463.1	Uncultured alpha proteobacterium clone RKPE90	Uncultured sponge associated bacterium clone en1492-89	99

RKPE91	JN411800.1	Uncultured gamma proteobacterium clone RKPE91	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE93	JX266014.1	Uncultured bacterium clone RKPE93	Uncultured Hawaiian water-associated SAR11 bacterium clone HF0010_09016	99
RKPE95	JN796453.1	Uncultured alpha proteobacterium clone RKPE95	Uncultured alpha proteobacterium (AB294937)	97
RKPE96	JX266010.1	Uncultured bacterium clone RKPE96	Uncultured marine bacterium clone SC5-101 (FN43560.1)	99
RKPE98	JN411742.1	Uncultured actinomycetales bacterium clone RKPE98	*Mycobacterium tuberculosis* (NR_044826.1); (Uncultured bacterium isolate DGGE gel band 4 (JN863714.1))	99
RKPE99	JN411730.1	Uncultured bacterium clone RKPE99	Uncultured bacterium clone ST13_70m_clone1 (HQ015557.1)	98
RKPE100	JN411744.1	Uncultured actinomycetales bacterium RKPE100	*Streptomyces flavovirens* strain CGMCC 4.575 (JQ924389.1)	99
RKPE101	JN411731.1	Uncultured proteobacterium clone RKPE101	Uncultured SAR11 cluster alpha proteobacterium clone 124	99
RKPE103	JN411749.1	Uncultured bacterium clone RKPE103	Uncultured coral-associated bacterium clone Apal_K23 (GU118137.1)	94
RKPE105	JN411790.1	Uncultured proteobacterium clone RKPE105	Uncultured bacterium clone Reef_P09 (GU119233.1)	97
RKPE110	JN411804.1	Uncultured proteobacterium clone RKPE110	Uncultured bacterium clone Gven_G10 (GU118518.1)	98
RKPE112	JN411779.1	Uncultured firmicutes bacterium clone RKPE112	*Staphylococcus pseudointermedius* LMG 22219 (NR_042284.1)	99
RKPE114	JN411737.1	Uncultured proteobacterium clone RKPE114	Uncultured coral associated bacterium clone BT60DS1BE12	94
RKPE115	JN411736.1	Uncultured proteobacterium clone RKPE115	Uncultured healthy coral associated bacterium clone SHFH663	98
RKPE120	JN796466.1	Uncultured alpha proteobacterium clone RKPE120	Uncultured bacterium clone SHFH457 (FJ203421.1)	96
RKPE121	JN411762.1	Uncultured bacterium clone RKPE121	Uncultured organism clone Apal_J16 (GU119563.1)	94
RKPE091	JN411800.1	Uncultured gamma proteobacterium clone RKPE91	Uncultured octocoral associated bacterium clone C9 (JQ691583.1)	98
RKPE107	JX266011.1	Uncultured bacterium clone RKPE107	Uncultured stony coral associated bacterium JQ347383.1	99
RKPE123	JN411789.1	Uncultured proteobacterium clone RKPE123	Uncultured hard coral bacterium clone AP37 (JQ347329.1)	99
RKPE128	JN796454.1	Uncultured alpha proteobacterium clone RKPE128	Uncultured microbial mat bacterium (FN663015.1)	98
RKPE130	JN411780.1	Uncultured bacteroidetes bacterium RKPE130	Uncultured coral associated bacterium cl. MHH03 (FJ216111.1)	95
RKPE132	JN411765.1	Uncultured cyanobacterium clone RKPE132	Uncultured bacterium clone BM89PA1BbC9	97
RKPE138	JN796457.1	Uncultured alpha proteobacterium clone RKPE138	*Porphyrobacter dokdonensis* (NR_043469.1)	99
RKPE143	JN796456.1	Uncultured alpha proteobacterium clone RKPE143	Uncultured microbial mat bacterium (FN663015.1)	98
RKPE144	JN411743.1	Uncultured actinomycetales bacterium clone RKPE144	*Mycobacterium tuberculosis* (NR_044826.1); (Uncultured bacterium isolate DGGE gel band 4 (JN863714.1)	99
RKPE145	JN411791.1	Uncultured proteobacterium clone RKPE145	Uncultured bacterium clone 6C232341 (EU804424.1)	99

RKPE150	JN411750.1	Uncultured bacterium clone RKPE150	Uncultured surface marine bacterium (FM214293.1)	90
RKPE152	JN411751.1	Uncultured bacterium clone RKPE152	Uncultured bacterium clone APM21 (JQ347383.1)	98
RKPE153	JN411745.1	Uncultured bacterium clone RKPE153	Uncultured sponge associated bacterium clone BAC-E110 (JN113072.1)	97
RKPE157	JN411787.1	Uncultured proteobacterium clone RKPE157	Uncultured coral associated bacterium clone Apal_N19 (GU118096.1)	98
RKPE168	JN796458.1	Uncultured alpha proteobacterium clone RKPE168	Uncultured hard coral associated bacterium clone SHFG464	98
RKPE170	JN796469.1	Uncultured alpha proteobacterium clone RKPE170	Uncultured diseased stony coral tissue bacterium clone SHFG464 (FJ203077.1)	98
RKPE176	JN796470.1	Uncultured alpha proteobacterium clone RKPE176	*Rickettsia rhipicephali* strain 3-7-6 (NR_025921.1)	99
RKPE185	JN411738.1	Uncultured alpha proteobacterium clone RKPE185	Uncultured coral associated bacterium clone BT60DS1BB10 (AF365685.1)	99
RKPE219	JN411777.1	Uncultured firmicutes bacterium clone RKPE219	*Staphylococcus aureus* subsp. aureus strain S33 R (NR_037007.1)	99
RKPE221	JN411739.1	Uncultured actinomycetales bacterium clone RKPE221	*Brevibacterium* sp. EP11 (AM398220.1)	99
RKPE223	JN411752.1	Uncultured bacterium clone RKPE223	Uncultured bacterium clone Gven_K23 (GU118339.1); Uncultured bacterium isolate DGGE gel band 1 (JN863711.1)	99
RKPE225	JN411776.1	Uncultured firmicutes bacterium clone RKPE225	Uncultured coral associated bacterium clone BM89DS6BbE6 (AF365668.1)	99
RKPE230	JN796451.1	Uncultured alpha proteobacterium clone RKPE230	*Bradyrhizobium betae* strain PL7HG1 (NR_029104.1)	98
RKPE245	JN411793.1	Uncultured gamma proteobacterium clone RKPE245	*Pseudomonas aeruginosa* strain R1-73 (JQ659528.1)	99
RKPE250	JN411753.1	Uncultured bacterium clone RKPE250	Uncultured bacterium clone Gven_K23 (GU118339.1); Uncultured bacterium isolate DGGE gel band 1 (JN863711.1)	98 - 99
RKPE253	JN411778.1	Uncultured firmicutes bacterium clone RKPE253	*Streptococcus salivarius* strain ATCC 7073 (NR_042776.1)	98
RKPE259	JN796471.1	Uncultured alpha proteobacterium clone RKPE259	*Rickettsia rhipicephali* strain 3-7-6 (NR_025921.1)	99
RKPE280	JX266015.1	Uncultured firmicutes clone RKPE280	Uncultured bacterium clone ncd2286g06c1 (JF196886.1)	99
RKPE289	JN411754.1	Uncultured bacterium clone RKPE289	*Coxiella* endosymbiont of American tick (AY939824.1)	98
RKPE290	JN796472.1	Uncultured alpha proteobacterium clone RKPE290	Uncultured disease coral associated tissue bacterium clone SHFG464 (FJ203077.1)	98
RKPE311	JN411755.1	Uncultured bacterium clone RKPE311	Uncultured bacterium clone ncd763f02c1 (HM299723.1)	94
	Indicates high sequence identity (≥98% sequence similarity) to other octocoral-associated 16S rRNA gene sequences in GenBank			

APPENDIX B

Table B1: List of dilutions used for *P. elisabethae* samples for A) Bimini Site 1 and B) Bimini site 2. BS1 refers to Bimini Site 1 (Victory Reef), and BS2 refers to Bimini Site 2 (Tuna Alley); A, B, and C refer to coral replicates collected at each site, while 104 and 51 refer to screen sizes, and PF refers to particle filtrate.

A)

Sample	Dilution 1	Dilution 2	µL/well	Comments
PE-BS1-A – 104µm	Undiluted	1/10	20	
PE-BS1-A – 51 µm	Undiluted	1/2	20	
PE-BS1-A-PF	1/10	1/1000	20	
PE-BS1-B – 104 µm	Undiluted	Undiluted	20	
PE-BS1-B – 51 µm	Undiluted	Undiluted	20	
PE-BS1-B-PF	1/10	1/1000	20	
PE-BS1-C – 104 µm	Undiluted	1/2	20	Greater mucus content than other samples
PE-BS1-C – 51 µm	Undiluted	1/2	20	
PE-BS1-C-PF	1/10	1/1000	20	

B)

Sample	Dilution 1	Dilution 2	µL/well	Comments
PE-BS2-A – 104µm	Undiluted	1/50	10	
PE-BS2-A – 51 µm	1/10	1/500	10	
PE-BS2-A-PF	1/100	1/1000	10	
PE-BS2-B – 104 µm	Undiluted	1/50	10	Greater mucus content than other samples
PE-BS2-B – 51 µm	1/10	1/500	10	
PE-BS2-B-PF	1/100	1/1000	10	
PE-BS2-C – 104 µm	Undiluted	1/50	10	
PE-BS2-C – 51 µm	1/10	1/500	10	
PE-BS2-C-PF	1/100	1/1000	10	

Table B2: GenBank accession numbers for bacteria cultivated from *P. elisabethae*. Replicate and Filters and replicates are applicable only to 2009-collected corals. Site 1, Victory Reef; Site 2, Tuna Alley. (2009); SS, San Salvador (2008).

Isolate	Organism	Acc No.	Site	Replicate (2009)	Particle size (2009)
V8	*Microbacterium* sp. isolate RKVR_V8	JX317705	1	A	PF
RKVR37	*Bacillus* sp. isolate RKVR37	JX317714	1	B	51
B20	*Vibrio* sp. isolate B20	JX407128	1	C	51
B27	*Pseudoalteromonas* sp. isolate B27	JX407132	1	C	51
B3	*Pseudoalteromonas* sp. isolate B3	JX407133	1	B	51
B30	*Ruegeria* sp. isolate B30	JX407134	1	B	PF
B36	*Pseudoalteromonas* sp. isolate B36	JX407135	1	C	51
B48	*Alteromonas* sp. isolate B48	JX407136	1	A	51
B6	*Pseudoalteromonas* sp. isolate B6	JX407138	1	A	PF
B9-1	*Pseudoalteromonas* sp. isolate B9-1	JX407141	1	C	104
B9-2	*Pseudoalteromonas* sp. isolate B9-2	JX407142	1	C	104
C8	*Alteromonas* sp. isolate C8	JX407147	1	A	51
D1	*Ruegeria* sp. isolate D1	JX407149	1	C	51
D2	*Vibrio* sp. isolate D2	JX407160	1	B	PF
D27	*Ruegeria* sp. isolate D27	JX407162	1	A	PF
D28	*Ruegeria* sp. isolate D28	JX407163	1	B	PF
D3	*Vibrio* sp. isolate D3	JX407164	1	B	51
D41	*Vibrio* sp. isolate D41	JX407170	1	A	51
D42	*Pseudoalteromonas* sp. isolate D42	JX407171	1	C	51
D47	*Vibrio* sp. isolate D47	JX407172	1	A	PF
D50_W	*Ruegeria* sp. isolate D50_W	JX407175	1	B	51
D55	*Vibrio* sp. isolate D55	JX407180	1	B	51
D59	*Vibrio* sp. isolate D59	JX407181	1	A	51
D6	*Vibrio* sp. isolate D6	JX407182	1	C	PF
D62	*Ruegeria* sp. isolate D62	JX407184	1	B	51
D68	*Vibrio* sp. isolate D68	JX407186	1	B	51
D7	*Vibrio* sp. isolate D7	JX407187	1	B	PF
D74	*Vibrio* sp. isolate D74	JX407190	1	B	51
D77	*Vibrio* sp. isolate D77	JX407192	1	B	51
D78	*Vibrio* sp. isolate D78	JX407193	1	C	PF
D81	*Pseudoalteromonas* sp. isolate D81	JX407195	1	B	51
G11	*Ruegeria* sp. isolate G11	JX407197	1	C	PF
G20	*Pseudoalteromonas* sp. isolate G20	JX407200	1	C	51
G24	*Ruegeria* sp. isolate G24	JX407202	1	A	51

G29	*Ruegeria* sp. isolate G29	JX407205	1	C	51
G3	*Vibrio* sp. isolate G3	JX407206	1	B	51
G5	*Pseudoalteromonas* sp. isolate G5	JX407207	1	A	104
G6	*Ruegeria* sp. isolate G6	JX407208	1	A	PF
G7	*Ruegeria* sp. isolate G7	JX407209	1	C	PF
G8	*Ruegeria* sp. isolate G8	JX407210	1	B	PF
U1	*Pseudoalteromonas* sp. isolate U1	JX407216	1	A	51
U5	*Ruegeria* sp. isolate U5	JX407217	1	A	PF
U11	*Shewanella* sp. isolate U11	JX407218	1	B	51
V5	*Pseudoalteromonas* sp. isolate V5	JX407221	1	B	51
V1	*Vibrio* sp. isolate V1	JX407222	1	A	PF
RKVR02	*Pseudoalteromonas* sp. isolate RKVR02	JX407229	1	C	PF
RKVR03	*Pseudoalteromonas* sp. isolate RKVR03	JX407230	1	A	PF
RKVR04	*Pseudoalteromonas* sp. isolate RKVR04	JX407231	1	A	
RKVR07	*Pseudoalteromonas* sp. isolate RKVR07	JX407233	1	A	51
RKVR13	*Pseudoalteromonas* sp. isolate RKVR13	JX407238	1	C	51
RKVR16	*Ruegeria* sp. isolate RKVR16	JX407240	1	C	51
RKVR30	*Ruegeria* sp. isolate RKVR30	JX407244	1	A	51
RKVR33	*Ruegeria* sp. isolate RKVR33	JX407245	1	C	51
RKVR34	*Pseudoalteromonas* sp. isolate RKVR34	JX407246	1	C	51
RKVR36	*Bacillus* sp. isolate RKVR36	JX407247	1	A	PF
RKVR39	*Vibrio* sp. isolate RKVR39	JX407248	1	B	51
RKVR40	*Pseudoalteromonas* sp. isolate RKVR40	JX407249	1	C	51
RKVR41	*Pseudooidiomarina* sp. isolate RKVR41	JX407250	1	A	51
RKVR42	*Microbacterium* sp. isolate RKVR42	JX407251	1	A	PF
Ps1E1	*Arthrobacter* sp. isolate RKVR_Ps1E1	JX317703	1	NA	PF
B28	*Bacillus* sp. isolate RKVR_B28	JX317686	2	A	PF
G2	*Aquimarina* sp. isolate RKVR_G2	JX317687	2	B	51
E3	*Aquimarina* sp. isolate RKVR_E3	JX317688	2	B	51
B16-1	*Bacillus* sp. isolate RKVR_B16-1	JX317689	2	A	PF
D43	*Kytococcus* sp. isolate RKVR_D43	JX317690	2	C	51
E5	*Macrococcus* sp. isolate RKVR_E5	JX317691	2	A	51
F1	*Bacillus* sp. isolate RKVR_F1	JX317692	2	A	PF
F3	*Bacillus* sp. isolate RKVR_F3	JX317693	2	A	PF
F4	*Bacillus* sp. isolate RKVR_F4	JX317694	2	A	PF
F5	*Bacillus* sp. isolate RKVR_F5	JX317695	2	C	PF
D22	*Microbacterium* sp. isolate RKVR_D22	JX317696	2	C	PF
D73	*Marinilactibacillus* sp. isolate RKVR_D73	JX317697	2	B	51
D69	*Bacillus* sp. isolate RKVR_D69	JX317698	2	B	104
G17	*Bacillus* sp. isolate RKVR_G17	JX317699	2	A	51
U3	*Bacillus* sp. isolate RKVR_U3	JX317700	2	B	PF
L1	*Microbacterium* sp. isolate RKVR_L1	JX317701	2	C	51
L3	*Micrococcus* sp. isolate RKVR_L3	JX317702	2	C	PF

V2	*Bacillus* sp. isolate RKVR_V2	JX317704	2	A	PF
V12	*Janibacter* sp. isolate RKVR_V12	JX317706	2	A	PF
RKVR11	*Janibacter* sp. isolate RKVR11	JX317707	2	A	PF
RKVR17	*Micrococcus* sp. isolate RKVR17	JX317708	2	B	104
RKVR18	*Bacillus* sp. isolate RKVR18	JX317709	2	B	104
RKVR25	*Aquimarina* sp. isolate RKVR25	JX317710	2	C	51
RKVR26	*Macrococcus* sp. isolate RKVR26	JX317711	2	A	51
RKVR38	*Bacillus* sp. isolate RKVR38	JX317715	2	C	PF
RKVR31	*Paraliobacillus* sp. isolate RKVR31	JX317716	2	A	PF
RKVR24	*Aquimarina* sp. isolate RKVR24	JX317743	2	A	PF
RKVR35	*Sphingopyxis* sp. isolate RKVR35	JX407110	2	A	PF
RKVR_B7	*Amphritea* sp. isolate B7	JX407111	2	A	51
RKVR_L4	*Amphritea* sp. isolate L4	JX407112	2	A	51
RKVR_C15	*Sphingopyxis* sp. isolate C15	JX407113	2	A	PF
RKVR_V14	*Sphingomonas* sp. isolate V14	JX407114	2	B	51
RKVR21	*Vibrio* sp. isolate RKVR21	JX407116	2	B	PF
A10	*Vibrio* sp. isolate A10	JX407117	2	A	PF
A12	*Vibrio* sp. isolate A12	JX407118	2	A	PF
A2	*Vibrio* sp. isolate A2	JX407119	2	A	PF
A4	*Vibrio* sp. isolate A4	JX407120	2	A	PF
A5	*Vibrio* sp. isolate A5	JX407121	2	A	PF
A6	*Vibrio* sp. isolate A6	JX407122	2	A	PF
A7	*Vibrio* sp. isolate A7	JX407123	2	A	PF
A8	*Vibrio* sp. isolate A8	JX407124	2	A	51
B10	*Pseudoalteromonas* sp. isolate B10	JX407125	2	A	51
B14	*Pseudoalteromonas* sp. isolate B14	JX407126	2	B	51
B2	*Pseudoalteromonas* sp. isolate B2	JX407127	2	B	51
B23	*Vibrio* sp. isolate B23	JX407129	2	B	104
B24	*Pseudoalteromonas* sp. isolate B24	JX407131	2	B	104
B5	*Pseudoalteromonas* sp. isolate B5	JX407137	2	B	51
B7-1	*Ruegeria* sp. isolate B7-1	JX407139	2	A	51
B7-2	*Pseudoalteromonas* sp. isolate B7-2	JX407140	2	A	51
C13	*Alteromonas* sp. isolate C13	JX407143	2	B	51
C2	*Alteromonas* sp. isolate C2	JX407144	2	C	PF
C4	*Alteromonas* sp. isolate C4	JX407146	2	B	51
C9	*Pseudoalteromonas* sp. isolate C9	JX407148	2	B	PF
D10	*Pseudoalteromonas* sp. isolate D10	JX407150	2	B	104
D12	*Vibrio* sp. isolate D12	JX407152	2	B	51
D13	*Vibrio* sp. isolate D13	JX407153	2	B	104
D14	*Vibrio* sp. isolate D14	JX407154	2	A	104
D15	*Vibrio* sp. isolate D15	JX407155	2	B	PF
D16	*Vibrio* sp. isolate D16	JX407156	2	B	PF
D17	*Vibrio* sp. isolate D17	JX407157	2	B	51

D18	*Vibrio* sp. isolate D18	JX407158	2	B	PF
D19	*Vibrio* sp. isolate D19	JX407159	2	B	51
D20	*Vibrio* sp. isolate D20	JX407161	2	B	51
D33	*Pseudoalteromonas* sp. isolate D33	JX407166	2	C	PF
D37	*Vibrio* sp. isolate D37	JX407167	2	B	51
D48	*Alteromonas* sp. isolate D48	JX407173	2	A	51
D5	*Vibrio* sp. isolate D5	JX407174	2	B	51
D51_W	*Pseudoalteromonas* sp. isolate D51-W	JX407176	2	B	51
D51_Y	*Pseudoalteromonas* sp. isolate D51_Y	JX407177	2	B	51
D52	*Alteromonas* sp. isolate D52	JX407178	2	A	PF
D53	*Pseudoalteromonas* sp. isolate D53	JX407179	2	C	PF
D66	*Vibrio* sp. isolate D66	JX407185	2	A	PF
D71	*Photobacterium* sp. isolate D71	JX407188	2	A	PF
D72	*Vibrio* sp. isolate D72	JX407189	2	B	PF
D75	*Vibrio* sp. isolate D75	JX407191	2	B	51
D80	*Alteromonas* sp. isolate D80	JX407194	2	A	PF
D9	*Vibrio* sp. isolate D9	JX407196	2	B	PF
G18	*Vibrio* sp. isolate G18	JX407199	2	B	PF
G22	*Ruegeria* sp. isolate G22	JX407201	2	A	PF
G25	Xanthomonadaceae isolate G25	JX407203	2	B	51
G28	*Ruegeria* sp. isolate G28	JX407204	2	A	51
H2	*Vibrio* sp. isolate H2	JX407212	2	C	PF
H3	*Vibrio* sp. isolate H3	JX407213	2	B	104
H5	*Vibrio* sp. isolate H5	JX407215	2	B	PF
V3	*Pseudoalteromonas* sp. isolate V3	JX407219	2	B	51
V4	*Pseudoalteromonas* sp. isolate V4	JX407220	2	B	51
V7	*Alteromonas* sp. isolate V7	JX407223	2	C	PF
V9	*Pseudoalteromonas* sp. isolate V9	JX407224	2	B	51
V10	Rhodobacteraceae sp. isolate V10	JX407225	2	A	PF
V11	*Photobacterium* sp. isolate V11	JX407226	2	C	PF
V13	*Alteromonas* sp. isolate V13	JX407227	2	B	51
RKVR01	*Vibrio* sp. isolate RKVR01	JX407228	2	A	PF
RKVR05	*Pseudoalteromonas* sp. isolate RKVR05	JX407232	2	A	51
RKVR08	*Alteromonas* sp. isolate RKVR08	JX407234	2	A	104
RKVR09	*Alteromonas* sp. isolate RKVR09	JX407235	2	B	51
RKVR15	*Photobacterium* sp. isolate RKVR15	JX407239	2	A	PF
RKVR19	*Vibrio* sp. isolate RKVR19	JX407241	2	C	PF
RKVR20	*Pseudoalteromonas* sp. isolate RKVR20	JX407242	2	C	PF
RKVR27	*Paracoccus* sp. isolate RKVR27	JX407243	2	B	51
RKVR43	*Vibrio* sp. isolate RKVR43	JX407252	2	A	PF
RKVR14	*Pseudoalteromonas* sp. isolate RKVR14	JX407253	2	A	51
RKVR06	*Vibrio* sp. isolate RKVR06	JX407254	2	A	PF
RKVR22	*Shewanell*a sp. isolate RKVR22	JX407255	2	A	51

RKVR23	*Alteromonas* sp. isolate RKVR23	JX407256	2	C	PF
RKVR28	*Marinilactibacillus* sp. isolate RKVR28	JX407257	2	A	PF
RKVR29	*Micrococcus* sp. isolate RKVR29	JX317712	2	NA	PF
RKVR32	*Janibacter* sp. isolate RKVR32	JX317713	2	B	51
RKVR_L5	*Pseudoalteromonas* sp. isolate L5	JX407115	2	C	51
D30	*Vibrio* sp. isolate D30	JX407165	2	A	51
G13	*Alteromonas* sp. isolate G13	JX407198	2	A	51
RKVR10	*Alteromonas* sp. isolate RKVR10	JX407236	2	C	51
C3	*Alteromonas* sp. isolate C3	JX407145	2	A	PF
D11	*Vibrio* sp. isolate D11	JX407151	2	A	PF
D61	*Alteromonas* sp. isolate D61	JX407183	2	A	PF
H4	*Vibrio* sp. isolate H4	JX407214	2	A	PF
RKVR12	*Pseudoalteromonas* sp. isolate RKVR12	JX407237	2	A	PF
D39	*Vibrio* sp. isolate D39	JX407168	NA	A	PF
D40	*Vibrio* sp. isolate D40	JX407169	NA	A	PF
H1	*Vibrio* sp. isolate H1	JX407211	PEA	A	PF
SS1	*Staphylococcus* sp. isolate RKVR_SS1	JX317679	SS		
SS2	*Nocardioides* sp. isolate RKVR_SS2	JX317680	SS		
SS3	*Micrococcus* sp. isolate RKVR_SS3	JX317681	SS		
SS5	*Nocardioides* sp. isolate RKVR_SS5	JX317682	SS		
SS12	*Oceanobacillus* sp. isolate RKVR_SS12	JX317683	SS		
SS14	*Bacillus* sp. isolate RKVR_SS14	JX317684	SS		
SS12	*Staphylococcus* sp. isolate RKVR_SS12	JX317685	SS		
RKBH-B54	*Streptomyces* sp. isolate RKBH-B54	JX317717	SS		
RKBH-B109	*Micrococcus* sp. isolate RKBH-B109	JX317718	SS		
RKBH-B100	*Microbacterium* sp. isolate RKBH-B100	JX317719	SS		
RKBH-B58	*Brevibacterium* sp. isolate RKBH-B58	JX317720	SS		
RKBH-B62	*Nocardiopsis* sp. isolate RKBH-B62	JX317721	SS		
RKBH-B150	*Terribacillus* sp. isolate RKBH-B150	JX317722	SS		
RKBH-B102	*Rhodococcus* sp. isolate RKBH-B102	JX317723	SS		
RKBH-B86	*Pseudonocardia* sp. isolate RKBH-B86	JX317724	SS		
RKBH-B104	*Rhodococcus* sp. isolate RKBH-B104	JX317725	SS		
RKBH-B74	*Staphylococcus* sp. isolate RKBH-B74	JX317726	SS		
RKBH-B106	*Bacillus* sp. isolate RKBH-B106	JX317727	SS		
RKBH-B108	*Planococcus* sp. isolate RKBH-B108	JX317728	SS		
RKBH-B152	*Streptomyces* sp. isolate RKBH-B152	JX317729	SS		
RKBH-	*Bacillus* sp. isolate RKBH-B153	JX317730	SS		

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RKBH-B55	*Bacillus* sp. isolate RKBH-B55	JX317731	SS
RKBH-B75	*Microbacterium* sp. isolate RKBH-B75	JX317732	SS
RKBH-B65	*Staphylococcus* sp. isolate RKBH-B65	JX317733	SS
RKBH-B113	*Gordonia* sp. isolate RKBH-B113	JX317734	SS
RKBH-B114	*Bacillus* sp. isolate RKBH-B114	JX317735	SS
RKBH-B80	*Exiguobacterium* sp. isolate RKBH-B80	JX317736	SS
RKBH-B56	*Staphylococcus* sp. isolate RKBH-B56	JX317737	SS
RKBH-B115	*Kytococcus* sp. isolate RKBH-B115	JX317738	SS
RKBH-B116	*Bacillus* sp. isolate RKBH-B116	JX317739	SS
RKBH-B57	*Micrococcus* sp. isolate RKBH-B57	JX317740	SS
RKBH-B155	*Micrococcus* sp. isolate RKBH-B155	JX317741	SS
RKBH-B156	*Staphylococcus* sp. isolate RKBH-B156	JX317742	SS
RKBH-B103	*Roseomonas* sp. isolate RKBH-B103	JX407130	SS
RKBH-B110	*Pseudomonas* sp. isolate RKBH-B110	JX407258	SS
RKBH-B105	*Salinicola* sp. isolate RKBH-B105	JX407259	SS
RKBH-B107	*Enterobacter* sp. isolate RKBH-B107	JX407260	SS
RKBH-B001	*Paracoccus* sp. isolate RKBH-B001	JX407261	SS
SS9	*Ruegeria* sp. isolate SS9	JX407262	SS
SS17	*Cohaesibacter* sp. isolate SS17	JX407263	SS